U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

CARDIAC MORPHOLOGICAL CHANGES INDUCED BY LOW-FREQUENCY NOISE IN RATS

EDUARDO JOSÉ PEREIRA ANTUNES

Tese de doutoramento em Ciências Médicas

2013

EDUARDO JOSÉ PEREIRA ANTUNES

Cardiac Morphological Changes induced by Low-Frequency Noise in Rats

(Alterações Morfológicas Cardíacas induzidas por Ruído de Baixa Frequência, no Rato)

Tese de Candidatura ao grau de Doutor em Ciências Médicas submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientação científica pelo Professor Doutor José Martins dos Santos, professor catedrático do Instituto de Ciências da Saúde Egas Moniz e co-orientação pelo Professor Doutor Artur Águas, professor catedrático do Instituto de Ciências Biomédicas de Abel Salazar.

To Rosária and Orlando

To Pedro, Mariana and Sandra

“You will never do anything in this world without courage. It is the greatest quality of the mind next to honor”

Aristotle

List of publications included in this thesis

Antunes E, Oliveira P, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Histomorphometric evaluation of the coronary arterial vessels in rats submitted to industrial noise. Acta Cardiol 2013; 68: 285 – 289.

Antunes E, Oliveira P, Borrecho G, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Myocardial fibrosis in rats exposed to low frequency noise. Acta Cardiol 2013; 68: 241 – 245.

Antunes E, Borrecho G, Oliveira P, Brito J, Águas A, Martins dos Santos J. Immunohistochemical evaluation of cardiac connexin43 in rats exposed to low-frequency noise. Int J Clin Exp Pathol; 2013; 6: 1874 – 1879.

Antunes E, Borrecho G, Oliveira P, Alves de Matos AP, Brito J, Águas A, Martins dos Santos J. Effects of low-frequency noise on cardiac collagen and cardiomyocyte ultrastructure: An immunohistochemical and electron microscopy study. Int J Clin Exp Pathol; (Accepted 2013).

CONTENTS

ACKNOWLEDGMENTS

First I wish to emphasize that I was very lucky for having as coordinator of this thesis a very special friend, whose friendship comes from the high school times. The art of knowledge and of teaching and the perseverance of his strong personality are some of his hallmarks. I would like to thank Professor José Martins dos Santos for all the support, help, contribution and conduction of this thesis and of course for the good friendship moments. So, I was lucky and lucky I am for having a great friend.

I would also like to thank Professor Artur Águas (Abel Salazar Institute for Biomedical Sciences) co-coordinator of this thesis for all the support, suggestions, collaboration and contribution to this work;

Special thanks to Professor Pedro Oliveira (Egas Moniz Institute for Health Sciences) for his friendship, help, suggestions, constant availability and contribution to this thesis;

Special thanks to the Assistant Pathologist Gonçalo Borrecho (Egas Moniz Institute for Health Sciences) for the hard work he had in the preparation of the samples and for his willingness for data collection and calculations. Without his help this work would not have been possible;

To Professor José Brito (Egas Moniz Institute for Health Sciences) I thank the statistic support and his always pertinent suggestions during the manuscript preparations;

To Professor Maria João Oliveira (Abel Salazar Institute for Biomedical Sciences) I thank her contribution for the development of this thesis;

To Professor António Alves de Matos (Egas Moniz Institute for Health Sciences) I thank his support in electron microscopy and suggestions for manuscripts;

To Dr. Castelo Branco I thank his suggestions and knowledge in the field;

I would also like to thank Professor Roland Stroobandt (Ghent University Hospital) for having followed our work since the first manuscript and for his collaboration during the development of this thesis;

I also thank the fantastic team of the department of Anatomy and colleagues of the Egas Moniz Institute for Health Sciences who always have been present in the difficult moments, Professor Carlos Zagalo, Professor José Grilo, Professor Vitor Tavares,

Professor José João Mendes, Dr. Alzira Cavacas, Dr. António José Sousa and Professor Jorge da Fonseca;

To Dr. Rui Ferreira, Director of the Department of Cardiology, Santa Marta Hospital I thank him all his willingness and contribution to reduce my assistant hospital work during parts of this thesis preparation;

To the other colleagues of the Department of Cardiology, Santa Marta Hospital, I thank them for all their contribution to reduce my hospital work during parts of this work;

I thank Professor Fernando Ferraz (Egas Moniz Institute for Health Sciences) for making available his department and microscopes in one of our studies;

I thank Dr. Mário Ferraz de Oliveira and Dr. Eugénia Pinto (São José Hospital) for their suggestions and collaboration during the development of our studies;

I thank the Assistant Pathologist Joana Couceiro (Egas Moniz Institute for Health Sciences) for her collaboration in one of our studies;

I thank the Assistant Pathologist Pedro Henriques (Egas Moniz Institute for Health Sciences) for his collaboration to this work;

I thank the Assistant Pathologists Rui Geraldes and Ana Lúcia Ponte (São José Hospital) for their collaboration in one of our studies;

I thank all the staff of the Egas Moniz Institute for Health Sciences who contributed to this thesis;

I thank Abel Salazar Institute for Biomedical Sciences and University of Porto for accepting me as a doctoral student;

And finally, special thanks to my close family. I had less time for them, but I always received their support, attention and help which allowed me to overcome many difficult moments and have a strong determination to complete this thesis.

ABSTRACT

Introduction: Low-frequency noise (LFN) can lead to structural and ultrastructural modifications in the extracellular matrix of several tissues, with an abnormal proliferation of collagen and development of fibrosis. In animal models, fibrosis was reported in lung parenchyma, tracheal epithelia, gastric mucosa, lymphatic vessels, arterial vessels and parotid gland among others after LFN exposure. In the heart, morphological changes were observed in cardiac valves and in the pericardium but without significant clinical consequences.

In this thesis we hypothesized that the new cardiac experimental model induced through LFN should be based on the vascular, myocardial and electrophysiological components whose alterations have unquestionable clinical implications. The importance of looking into the effects of LFN on the ventricular myocardium was the increased epidemiological evidence relating noise and hypertension and ischemic heart disease as well as the previous experimental studies showing fibrotic development of the extracellular matrix in several tissues.

The aim of this thesis was to evaluate cardiac morphological changes in the coronary artery vessels, myocardium and gap junctions induced by LFN and for that purpose, four consecutive studies with the use of two series of Wistar rats were performed.

Methods: In a series of 40 rats, a group with 20 rats exposed to industrial noise (IN) during a maximum period of 7 months and a group with 20 rats as age-matched controls, were considered for histomorphometric evaluation of the coronary artery vessels.

In other series of 46 rats, a group with 26 rats continuously exposed to LFN during a 3-month period and a group with 20 controls were considered for myocardial interstitial fibrosis evaluation. In a sub-group of 18 rats, 10 LFN-exposed rats and 8 controls were utilized for immunohistochemical analysis of connexin (Cx) 43. In a sub-group of 16 rats, 8 LFN-exposed and 8 control rats were considered for immunohistochemical evaluation of collagens I and III and in five rats the myocardial ultrastructure was analyzed with transmission electron microscopy (TEM).

In all series, the hearts were sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected. For immunohistochemical studies and evaluation of myocardial fibrosis, comparisons between the right ventricle, interventricular septum and left ventricle were performed.

Hematoxylin-eosin was used for histological observations of the arterial vessels and myocardium. Masson's trichrome staining was used to evaluate fibrosis in the coronary arteries and the chromotrope-aniline blue (CAB) staining was utilized to evaluate it in the myocardium. For the immunohistochemical evaluations, the rabbit polyclonal antibody Cx43 was used to analyze Cx43 and the polyclonal antibodies to collagen I and III were used for the collagens.

The computer *image J software* was used to analyze all morphological parameters evaluated by light microscopy. In 130 coronary artery vessels, the vessel caliber, the thickness of the wall and the perivascular dimensions were quantified and then the mean lumen-to-vessel wall and the mean vessel wall-to-perivascular tissue ratios were calculated. In 138 optical fields taken for myocardial fibrosis evaluation, the muscle and interstitial fibrosis were quantified and the mean fibrosis/muscle ratio was calculated. In 146 optical fields selected to evaluate Cx43, the mean Cx/muscle ratio was calculated after Cx43 and muscle had been quantified. A total of 132 optical fields were selected to evaluate type I collagen, type III collagen and the muscle and then the mean collagen I/muscle and collagen III/muscle ratios were calculated.

For ultrastructural analysis, with an illustrating purpose, ventricular ultrathin sections were stained with uranyl acetate and lead citrate and were examined and photographed with a transmission electron microscope.

Results: The histological observation of the coronary artery vessels showed prominent perivascular tissue and fibrotic development among IN-exposed rats. The histomorphometric analysis showed that the mean lumen-to-vessel wall ratio was 0.7297 and 0.6940 respectively in IN-exposed and control rats. The mean vessel wall-to-perivascular tissue ratio was 0.4923 and 0.5540 respectively in IN-exposed and control animals ($p < 0.01$).

The histological observation with CAB staining of the myocardium showed the presence of fibrosis predominantly in LFN-exposed rats. Fibrosis increased 97.5%, 81.5% and 83.7%, respectively in the left ventricle, interventricular septum and right ventricle, in exposed rats ($p < 0.001$). The mean fibrosis/muscle ratio in the left ventricle, interventricular septum and right ventricle was significantly higher in LFN-exposed rats ($p < 0.001$).

The immunohistochemical analysis of Cx43 showed significant differences between exposed and control rats ($p = 0.001$). The mean Cx43/muscle ratio decreased *in totum* 43.3% among LFN-exposed rats ($p = 0.009$). There was a decrease of 46.2%, 22.2% and

55.6% respectively in the left ventricle ($p = 0.008$), interventricular septum ($p = 0.301$) and right ventricle ($p = 0.004$).

The immunohistochemical evaluation of type I and III collagens showed significant differences between LFN-exposed and control rats. The collagen I/muscle and collagen III/muscle ratios i*n totum* respectively increased 80% ($p < 0.001$) and 57.4% ($p < 0.05$) in LFN-exposed rats.

In LFN-exposed animals the ultrastructural analysis of the myocardium by TEM showed an accumulation of collagen in the extracellular matrix. In the cardiomyocyte, cell membrane separation with no visible gap junctions in the interplicate regions of the intercalated discs, as well as a considerable number of enlarged mitochondria, were observed.

Conclusions: Our results showed periarterial and myocardial fibrotic development as well as a reduction of connexin43 and defined a new cardiac morphological model induced by LFN, based on three anatomical components.

Industrial noise induces perivascular structural modifications in coronary artery vessels which opens the possibility of ischemia related to changes on vessel distensibility.

The significant increase of myocardial fibrosis may suggest ventricular functional changes induced by low-frequency noise.

The reduction of connexin43 together with the alterations detected at the intercalated discs of the cardiomyocytes, strongly suggest the existence of a morphological arrhythmogenic substrate induced by low-frequency noise.

Thus, we put forward the hypothesis that low-frequency noise can be another cause for developing myocardial ischemia, heart failure and ventricular tachyarrhythmias, which opens new and promising paths for experimental and clinical research in these areas.

RESUMO

Introdução: O ruído de baixa frequência (RBF) pode induzir alterações em vários tecidos e órgãos, caracterizadas por modificações na matriz extracelular com proliferação anormal de colagénio e desenvolvimento de fibrose tecidular. Em modelos animais detectou-se um aumento de fibrose no parênquima pulmonar, epitélio traqueal, mucosa gástrica, vasos linfáticos, vasos arteriais e na glândula parótida entre outros após exposição ao RBF. No coração foram observadas modificações morfológicas nas válvulas cardíacas e no pericárdio mas sem consequências clínicas significativas.

Na presente tese admitimos como hipótese que o novo modelo cardíaco experimental induzido pelo RBF deveria ser baseado em três componentes anatómicos cujas alterações têm repercussões clínicas inquestionáveis: artérias coronárias, miocárdio e tecido eléctrico especializado. A importância de investigar os efeitos do RBF no miocárdio ventricular prende-se com a evidência epidemiológica crescente entre ruído e hipertensão arterial e doença coronária bem como com os estudos experimentais demonstrativos de desenvolvimento fibrótico da matriz extracelular em diversos tecidos.

O objectivo desta tese foi o de avaliar as alterações morfológicas cardíacas nas artérias coronárias, no miocárdio e nas *gap junctions.* Para tal efectuámos quatro estudos experimentais consecutivos usando duas séries de ratos Wistar.

Métodos: Numa série com 40 ratos, consideraram-se dois grupos de 20, um exposto a ruído industrial durante um período máximo de 7 meses e outro de controlo, para efeitos de avaliação histomorfométrica dos vasos arteriais coronários.

Numa segunda série de 46 ratos, um grupo de 26 animais foi exposto continuamente a RBF durante 3 meses e um grupo de 20 foi considerado controlo. Esta série foi usada para avaliação de fibrose intersticial miocárdica e dois sub-grupos de animais com 18 e 16 ratos foram considerados respectivamente para as avaliações com imunohistoquímica da conexina43 (Cx43) e dos colagénios I e III. No sub-grupo de 18 ratos, 10 animais foram submetidos ao RBF e 8 foram controlos. No sub-grupo de 16 animais, 8 foram expostos ao RBF e 8 foram controlos. Deste último sub-grupo, foi feita avaliação ultraestrutural em 5 ratos.

Nas duas séries, os corações foram seccionados transversalmente do ápice para a base e o segmento médio-ventricular foi seleccionado. Para a avaliação de fibrose miocárdica e com imunohistoquímica foram efectuadas comparações entre o ventrículo direito, septo interventricular e ventrículo esquerdo.

Utilizou-se a coloração de hematoxilina-eosina para observação histológica vascular e do miocárdio. O tricrómico de Masson foi utilizado para avaliar a fibrose nas artérias coronárias e a coloração chromotrope-aniline blue (CAB) no miocárdio. Para a avaliação imunohistoquímica da Cx43 usou-se o anticorpo policlonal de coelho anti Cx43 e utilizaram-se os anticorpos policlonais para o colagénio I e III na avaliação imunohistoquímica dos colagénios.

O programa informático *Image J* foi utilizado para analizar todos os parâmetros morfológicos avaliados por microscopia óptica. Em 130 vasos arteriais coronários, o calibre do vaso, a espessura da parede e as dimensões perivasculares foram quantificadas seguidas do cálculo das médias dos rácios lume/parede e parede/tecido perivascular. Em 138 campos histológicos usados para avaliação da fibrose do miocárdio, o músculo e a fibrose intersticial foram quantificados e a média do rácio fibrose/músculo calculada. Em 146 campos histológicos escolhidos para avaliar Cx43, a média do rácio Cx43/músculo foi calculada após a quantificação da Cx43 e do músculo. Um total de 132 campos histológicos foram seleccionados para quantificação dos colagénios I e III e do músculo e as médias dos rácios colagénio I/músculo e colagénio III/músculo foram calculados.

Para a análise ultraestrutural e como uma avaliação preliminar ilustrativa, efectuaram-se cortes ultrafinos corados com acetato de uranilo e citrato de chumbo que foram examinados e fotografados num microscópio electrónico de transmissão.

Resultados: As observações histológicas mostraram um tecido perivascular proeminente e com fibrose exuberante nos ratos expostos ao ruído industrial. A análise histomorfométrica mostrou que a média do rácio lume/parede foi de 0,7297 e de 0,6940, respectivamente nos ratos expostos ao ruído industrial e nos ratos de controlo. A média do rácio parede/tecido perivascular foi respectivamente 0,4923 e de 0,5540 nos animais expostos ao ruído industrial e nos animais de controlo ($p < 0,01$).

As observações histológicas com a coloração de *CAB* mostraram fibrose mais acentuada nos ratos expostos ao RBF. A avaliação de fibrose miocárdica mostrou um aumento de 97,5%, 81,5% e 83,7% respectivamente no ventrículo esquerdo, septo interventricular e no ventrículo direito dos ratos expostos ao RBF ($p < 0,001$). A média do rácio fibrose/músculo foi significativamente mais elevada no ventrículo esquerdo, septo interventricular e ventrículo direito dos ratos expostos ao RBF ($p < 0,001$).

A análise imunohistoquímica da Cx43 mostrou diferenças estatisticamente significativas entre os ratos expostos e os controlos ($p = 0,001$). A média do rácio Cx43/músculo

reduziu-se *in totum* 43,3% nos ratos expostos ao RBF ($p = 0,009$). Ocorreu uma redução de 46,2%, 22,2% e 55,6% respectivamente no ventrículo esquerdo ($p = 0,008$), septo interventricular ($p = 0,301$) e ventrículo direito ($p = 0,004$).

A avaliação imunohistoquímica dos colagénios I e III mostrou diferenças significativas entre os ratos expostos ao RBF e os controlos. As médias dos rácios colagénio I/músculo e colagénio III/músculo aumentaram *in totum* respectivamente 80% ($p < 0,001$) e 57,4% ($p < 0,05$).

A análise ultraestrutural por microscopia electrónica de transmissão mostrou nos animais expostos ao RBF uma acumulação de colagénio na matriz extracelular e uma separação das membranas celulares sem *gap junctions* visíveis nas regiões longitudinais dos discos intercalares bem como um número considerável de mitocôndrias de grandes dimensões nos cardiomiócitos.

Conclusões: Os nossos resultados mostraram um aumento da fibrose periarterial e miocárdica bem como uma redução da conexina43 e definiram um novo modelo morfológico cardíaco induzido pelo ruído de baixa frequência baseado em três componentes anatómicos.

O ruído industrial induz modificações perivasculares nas artérias coronárias o que abre a possibilidade de ocorrência de isquémia miocárdica induzida por alterações da distensibilidade dos vasos.

O aumento significativo de fibrose miocárdica, pode sugerir a ocorrência de alterações funcionais ventriculares induzidas pelo ruído de baixa frequência.

A redução da conexina43 bem como as alterações detectadas ao nível dos discos intercalares, sugerem fortemente a existência de um substrato morfológico arritmogénico induzido pelo ruído de baixa frequência.

Admitimos que o ruído de baixa frequência possa ser uma outra causa de isquémia miocárdica, insuficiência cardíaca e taquiarritmias ventriculares, o que abre novas e promissoras linhas de investigação experimental e clínica nestas áreas.

1. INTRODUCTION

General considerations

Low-frequency noise (LFN) is characterized by large pressure amplitude (≥ 90 dB SPL) and low frequency bands (≤ 500 Hz) and can lead to structural and ultrastructural modifications in the extracellular matrix of several organs, with an abnormal proliferation of collagen and development of tissue fibrosis (1). In animal models, after LFN exposure, fibrosis was already found in lung parenchyma, tracheal epithelia, gastric mucosa, lymphatic vessels, arterial vessels and parotid gland, among others (2-7).

In the heart, morphological changes were observed in cardiac valves and in the pericardium. Morphological changes of cardiac valves included thickening, calcification and/or restriction of leaflet movement, the mitral valve being the most affected (8). In the pericardium, changes included thickening and additional development of cell layers (9). Nevertheless, neither the observed changes in the valves nor the alterations found in the pericardium seemed to have clinical or hemodynamic consequences.

The importance of looking into the effects of LFN on the ventricular myocardium is the increased evidence relating noise and hypertension and ischemic heart disease (10, 11). With respect to aircraft noise and hypertension the relative risks range between 1.4 and 2.1 for subjects who live in highly exposed areas with an average sound pressure level in the range of 60-70 dB (A). In ischemic heart disease, prospective studies on road traffic noise showed relative risks ranging from 1.1 to 1.5 for noise levels above 65-70 dB(A). Results from these studies were based on sound pressure measured in dB(A), without stratification of the vibration frequency. However, these noises are rich in low-frequency components and infrasound as occurs with the noise of the textile industry.

There has been some evidence that the clinical impact of any morphological tissue alteration induced by LFN will be more accentuated in LFN-exposed professions such as aircraft cabin crew members, aeronautical maintenance technicians, airplane pilots, ship machinists and workers of textile industrial plants (12 - 14). Workers of textile industrial plants had increased respiratory infections and changes in respiratory function (13, 14). In a study conducted in aircraft technicians, three clinical stages were defined according to 2, 5 or more than 10 years of LFN-exposure but a relation with cardiac modifications was not established (15).

More recently a study conducted to evaluate the effects of wind turbines showed debilitating health effects suggesting an acoustic pressure pulsations etiology, not related

to the audible frequency spectrum (16), which enlarges the field to explore the effects of LFN exposure. In fact, in modern societies everybody is exposed to several sources of low-frequency noise from the air conditioned systems to industrial wind turbines among many others.

Taking into consideration the experimental effects of LFN on the extracellular matrix of several tissues, one could admit the occurrence of myocardial fibrosis after LFN exposure. The clinical consequences of its presence would be inherent to the deposition of collagen between the cardiomyocytes and around the cardiac vessels leading to myocardial stiffness, left ventricular dysfunction, modifications in coronary flow reserve and ventricular arrhythmias as demonstrated through experimental work of other cardiac pathologies (17 – 20).

On the other hand, changes in gap junctions characterized by a reduction in connexin43 expression seem to have arrhythmogenic consequences in diseased human myocardium (21 - 23). The evidence that a reduction of connexin43 expression is critical to increase the propensity for ventricular tachyarrhythmias was reported in several experimental studies (24 – 29) as well as a strong enhancement of arrhythmogenic vulnerability can be reached by the association of increased fibrosis with a reduction of connexin43 (30).

In this thesis we hypothesized that the new experimental model induced by LFN should be based on the vascular, myocardial and electrophysiological components (fig. 1) whose alterations have unquestionable clinical implications. We focused our attention on the evaluation of perivascular and myocardial fibrotic development as well as on connexin43 changes.

For the arterial vessels the sound signal was emitted by an analogue noise generator, amplified and frequency-filtered. The noise level was characterized by a wide spectrum of frequencies with an important component under 500 Hz (fig. 2). For the myocardium and *gap junctions* we used an analogue noise generator producing a noise spectrum with a predominant low-frequency component (fig. 3).

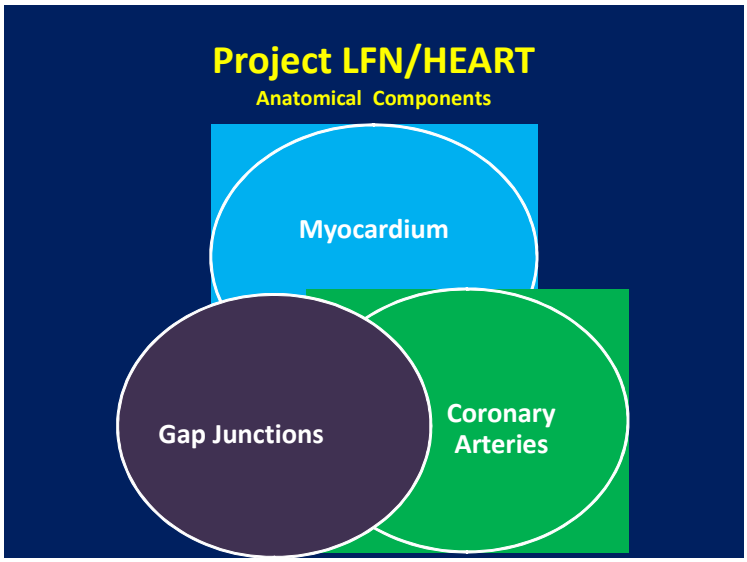


Figure 1 – Cardiac anatomical components for morphological evaluation of the LFN effects

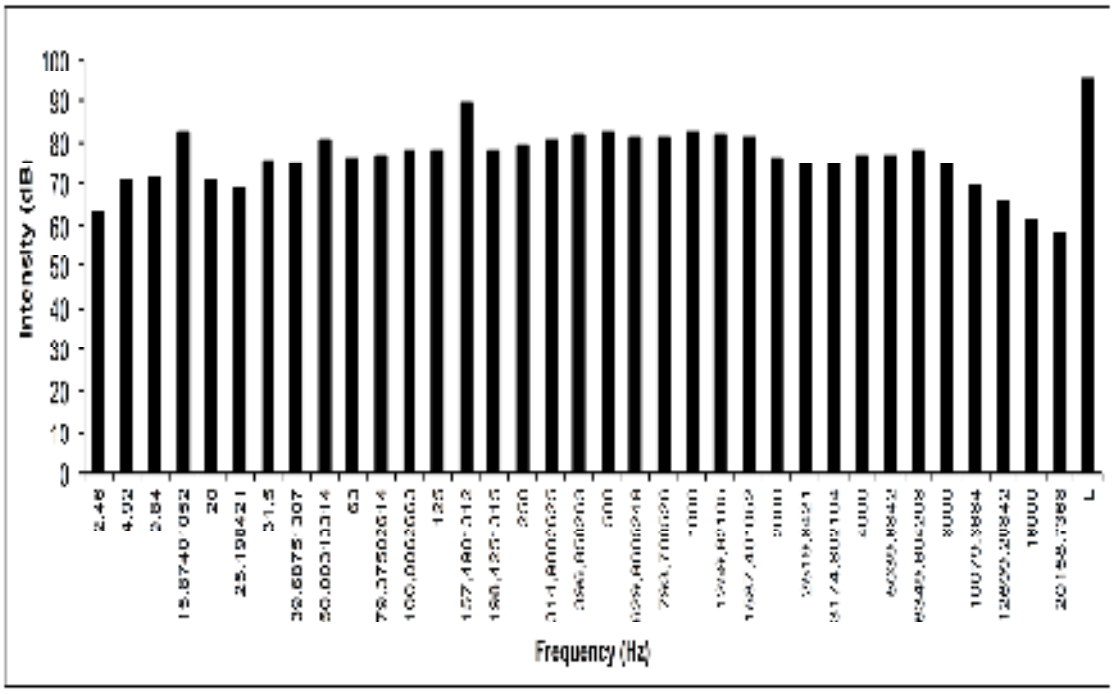


Figure 2 – Noise spectrum used for the coronary artery vessels

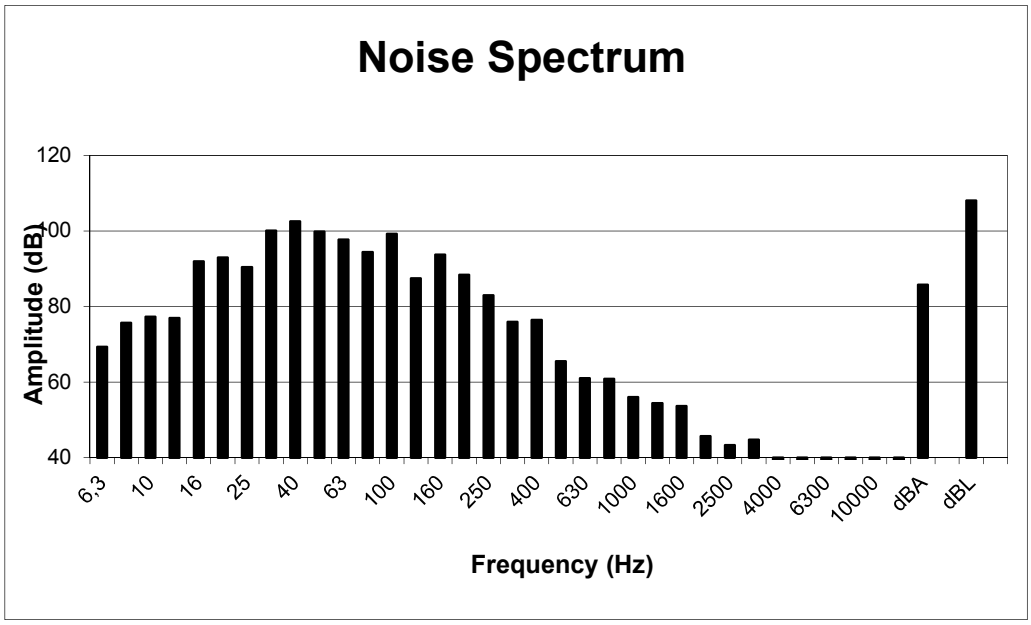


Figure 3 – Noise spectrum used for the myocardium and gap junctions

Myocardial and perivascular fibrosis

Fibroblasts are critical cells not only to normal myocardial function but also in the remodeling that occurs in response to pathological changes such as hypertension, myocardial infarction and heart failure (31 - 34). Many of the effects are mediated through the differentiation into myofibroblasts which exhibit proliferative, migratory and secretory properties and are responsive to proinflammatory cytokines, growth factors and vasoactive peptides as angiotensin II (31, 32).

Collagen produced by these cells has a crucial role, maintaining the myocardial structure and cardiac function, because of its contact proximity to cell and non-cell components of the myocardium. The extracellular matrix homeostasis requires a balance between the synthesis and degradation of the collagen and, changes in this interaction may result in an abnormal collagen network in the heart (33, 34).

Myocardial fibrosis reflects an excess of collagen in the extracellular matrix. In myocardial infarction its presence at sites remote to the necrotic area contributes to the development of cardiac remodeling and ventricular dysfunction (33). In hypertensive heart disease, myocardial fibrosis has an interstitial and perivascular location being the result of an exaggerated synthesis of collagen I and III and of a reduced activity of the metalloproteinases (34).

The occurrence of myocardial fibrosis can lead to regional electrophysiological heterogeneities that contribute to the genesis of atrial and ventricular arrhythmias (35, 36).

In some diseases, like hypertension or conditions with hemodynamic overload, fibroblasts are under mechanical stress and exhibit proliferative properties (37, 38). One can admit that the mechanical impact of LFN can have the same effects giving rise to an incorrect balance between the profibrotic and antifibrotic molecules.

The effects of LFN on the coronary artery vessels and on the myocardium are not known.

Intercalated discs and gap junctions

The intercalated disks are tridimensional structures distributed in the plicate regions (regions perpendicular to the long axis of the cells) and in the interplicate regions (parallel to the long axis of the cells) that include three junctional components: *Fasciae adherentes*, *gap junctions* and desmosomes (39, 40). These components are well organized in order to establish an appropriate mediation on the electrical and mechanical cardiomyocyte coupling (41).

The normal process of impulse conduction depends on the excitability of cardiomyocytes, on the electrical coupling between myocytes and on the network properties of cardiac tissue. The activation of adjacent cells is performed by the depolarizing current through the *gap junctions* which are composed by connexons that are hexamers of proteins called connexins (39, 42).

The cardiac electromechanical function depends on the structural integrity of the myocardium and the *gap junctions* constitute the intercellular transmission way of cardiac impulses from the sinoatrial node to the atrial and ventricular myocardium.

In the heart three main connexin isoforms are detected: Cx40, Cx43 and Cx45. Connexin40 is mainly expressed in the atrial myocytes, in the atrio-ventricular node and His-bundle. Connexin 43 is the most abundant and is distributed among atrial and ventricular cardiomyocytes and Purkinje fibers. Connexin 45 is mainly expressed in the sinoatrial node, atrio-ventricular node, His bundle and bundle branches (42).

Modifications concerning the number and distribution of *gap junctions* are observed in cardiac diseases showing myocardial necrosis and ventricular hypertrophy (43, 44). The reduction and lateralization of *gap junctions* can result in electrophysiological changes that

may lead to ventricular arrhythmias and to the development of ventricular dysfunction (41, 44-46).

Experimental studies showed a higher vulnerability for arrhythmias. The absence of connexin40 was observed with atrio-ventricular conduction disturbance (42). The heterogeneous reduction of connexin43 combined with increased collagen deposition and interstitial fibrosis enhances the vulnerability to ventricular arrhythmias (30).

It is not known whether LFN induces modifications on the cardiac electrophysiological milieu including the ventricular myocardium gap junctions.

Subject considerations and objectives

The effects of low frequency noise and infrasound on the extracellular matrix (1, 12, 47), the epidemiological evidence between noise and hypertension and ischemic heart disease (11), the gap junction remodeling occurring in several cardiac diseases (41, 46) and the reports of the effects of mechanical forces on cardiovascular collagen and gap junction remodeling (48, 49), make possible to admit the development of a new cardiac pathological model characterized by structural and ultrastructural changes induced by LFN.

An in depth acquired knowledge of the effects of LFN on the heart will be better reached if morphological studies regarding the evaluation of perivascular and myocardial fibrosis as well as the analysis of changes in gap junction are performed. Also, the evaluation of cardiac collagens and cardiomyocyte ultrastructure can add important information on cardiac effects of LFN and establish more possible links to new experimental and clinical studies.

Thus, the aim of this thesis was to characterize the morphological changes of the coronary artery vessels, myocardium and gap junctions induced by low-frequency noise in the rat heart.

For this purpose we evaluated two series of Wistar rats that were used in four consecutive studies. Some methodological aspects were common in all the studies as the selected ventricular fragment and the use of the *image J software* for morphometric evaluations.

In chapter 2 the histomorphometric analysis of the coronary artery vessels in rats submitted to industrial noise is presented. The quantification of interstitial fibrosis of the ventricular myocardium in rats exposed to low-frequency noise is demonstrated in chapter 3. An immunohistochemical evaluation of cardiac connexin43 in rats exposed to LFN is

the basis of chapter 4. The effects of LFN on type I and III collagens and cardiomyocyte ultrastructure are described in chapter 5. Chapter 6 is the final discussion and conclusions based on the results found in these studies.

References

1- Alves-Pereira M, Castelo Branco NAA. Vibroacoustic disease: biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signaling. Prog Bioph Mol Biol 2007; 93: 256 – 279.

2- da Fonseca J, dos Santos JM, Branco NC, Alves-Pereira M, Grande NR, Oliveira P, Martins AP. Noise-induced gastric lesions: a light and scanning electron microscopy study of the alterations of the rat gastric mucosa induced by low frequency noise. Cent Eur J Public Health 2006; 14: 35 - 38.

3- Grande NR, Águas AP, Sousa Pereira A, Monteiro E, Castelo Branco NAA. Morphological changes in the rat lung parenchyma exposed to low frequency noise. Aviat Space Environ Med 1999; 70: A70 - A77.

4- de Sousa Pereira A, Águas AP, Grande NR, Mirones J, Monteiro E, Castelo Branco NAA. The effect of chronic exposure to low frequency noise on rat tracheal epithelia. Aviat Space Environ Med. 1999; 70: A186 – A190.

5- Martins dos Santos J, Grande NR, Castelo Branco NAA, Zagalo C, Oliveira P, Alves-Pereira M. Lymphatic lesions and vibroacoustic disease. Eur J Lymphol 2004; 12: 17 – 20.

6- Oliveira PM, Pereira da Mata AD, Martins dos Santos JA, da Silva Marques DN, Branco NC, Silveira JM, Correia da Fonseca JC. Low-frequency noise effects on the parotid gland of the Wistar rat. Oral Dis 2007; 13: 468 – 473.

7- Martins dos Santos J, Grande NR, Castelo Branco NA, Zagalo C, Oliveira P. Vascular lesions and vibroacoustic disease. Eur J Anat 2002; 6: 17 – 21.

8- Marciniak W, Rodriguez E, Olszowska K, Atkov O, Botvin I, Araujo A, Pais F, Ribeiro CS, Bordalo A, Prazeres de Sá E, Ferreira D, Loureiro J, Castelo Branco MSN, Castelo Branco NAA. Echocardiographic evaluation in 485 aeronautical workers exposed to different noise environments. Aviat Space Environ Med 1999; 70 (3 suppl): A46 – A53.

9- Castelo Branco NAA, Águas AP, Pereira AS, Monteiro E, Fragata JIG, Tavares F, Grande NR. The human pericardium in vibroacoustic disease. Aviat Space Environ Med 1999; 70 (3 suppl): A54 – A62.

10- Babish W. Road traffic noise and cardiovascular risk. Noise & Health 2009; 10: 27 – 33.

11- Babish W. Transportation noise and cardiovascular risk: updated review and synthesis of epidemiological studies indicate that the evidence has increased. Noise & Health 2009; 8: 1 – 29.

12- Castelo-Branco NAA, Alves-Pereira M. Vibroacoustic disease. Noise & Health 2004; 6: 3 – 20.

13- Fishwick D, Fletcher AM, Pickering CA, McL Niven R, Faragher EB. Lung function in Lancashire cotton and man made fibre spinning mill operatives. Occup Environ Med 1996; 53: 46 – 50.

14- Cardoso AP, Oliveira MJ, Silva AM, Águas AP, Pereira AS. Effects of long term exposure to occupational noise on textile industry workers' lung function. Rev Port Pneumol 2006; 12: 45 – 59.

15- Castelo-Branco NAA. The clinical stages of vibroacoustic disease. Aviat Space Environ Med 1999; 70 (3 suppl): A32 – A39.

16- Ambrose SE, Rand RW. The Bruce McPherson infrasound and low frequency noise study. http://wndfo.net/D2795 (29th August 2013).

17- Weber KT, Brilla CG, Janicki JS. Myocardial fibrosis. Functional significance and regulatory factors. Cardiovasc Res 1993; 27: 13 – 31.

18- Assyang P, Carré F, Chevalier B, Delcayre C, Mansier P, Swynghedauw B. Compensated cardiac hypertrophy: arrhythmogenicity and the new myocardial phenotype. I. Fibrosis. Cardiovasc Res 1997; 34: 439 – 444.

19- Barasch E, Gottdiener JS, Aurigemma G, Kitzman DW, Han J, Kop WJ, Tracy RP. Association between elevated fibrosis markers and heart failure in the elderly. The cardiovascular health study. Circ Heart Fail 2009; 2: 303 – 310.

20- Isoyama S, Ito N, Satoh U, Takishima T. Collagen deposition and the reversal of coronary reserve in cardiac hypertrophy. Hypertension 1992; 20:491 - 500.

21- Peters NS, Coromilas J, Severs NJ, Wit AL. Disturbed connexin43 gap junction distribution correlates with the location of reentrant circuits in the epicardial border zone of healing canine infarcts that cause ventricular tachycardia. Circulation 1997; 97: 988 – 996.

22- Kawara T, Derksen R, de Groot JR, Coronel R, Tasseron S, Linnenbank AC, Hauer RN, Kirkels H, Janse MJ, de Bakker JM. Activation delay after premature stimulation in chronically diseased human myocardium relates to the architecture of interstitial fibrosis. Circulation 2001; 104: 3069 – 3075.

23- La Vecchia L, Ometto R, Bedogni F, Finocchi G, Mosele GM, Bozzola L, Bevilacqua P, Vincenzi M. Ventricular late potentials, interstitial fibrosis, and right ventricular function in patients with ventricular tachycardia and normal left ventricular function. Am J Cardiol 1998; 81: 790 – 792.

24- Gutstein DE, Morley GE, Tammaddon H, Vaidya D, Schneider MD, Chen J, Chien KR, Stuhlmann H, Fishman GI. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circ Res 2001; 88: 333 – 339.

25- Danik SB, Liu F, Zhang J, Suk HJ, Morley GE, Fishman GI, Gutstein DE. Modulation of cardiac gap junction expression and arrhythmic susceptibility. Circ Res 2004; 95: 1035 – 1041.

26- van Rijen HVM, Eckardt D, Degen J, Theis M, Ott T, Willecke K, Jonsma HJ, Opthof T, Bakker JMT. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. Circulation 2004; 109: 1048 – 1055.

27- Uzzaman M, Honjo H, Tagagishi Y, Emdad L, Magee AI, Severs NJ, Kodama I. Remodeling of gap junctional coupling in hypertrophied right ventricles of rats with monocrotaline-induced pulmonary hypertension. Circ Res 2000; 86: 871 – 879.

28- Mayama T, Matsumura K, Lin H, Ogawa K, Imanaga I. Remodeling of cardiac gap junction connexin 43 and arrhythmogenesis. Exp Clin Cardiol 2007; 12: 67 – 76.

29- Lin H, Ogawa K, Imanaga I, Tribulova N. Remodeling of connexin 43 in the diabetic rat heart. Mol Cell Biochem 2006; 290: 69 – 78.

30- Jansen JA, van Veen AA, Bosch AA, van der Nagel R, Vos MA, Bakker JM, van Rijen HV. Arrhythmia vulnerability of aged haploinsufficient Cx43 mice is determinant by heterogeneous downregulation of Cx43 combined with increased fibrosis. Circulation 2008; 118: S494.

31- Brilla CG, Scheer C, Rupp H. Renin-angiotensin system and myocardial collagen matrix: modulation of cardiac fibroblast function by angiotensin II type 1 receptor antagonism. J hypertens 1997; 15 (Suppl 6): S13 – S19.

32- Petrov VV, Fagard RH, Lijnen PJ. Stimulation of collagen production by transforming growth factor-β1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 2002; 39: 258 – 263.

33- van den Borne SWM, Diez J, Blankesteijn M, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 2010; 7: 30 – 37.

34- Diez J. Mechanisms of cardiac fibrosis in hypertension. J Clin Hypertens 2007; 9: 546 – 550.

35- Tribulová N, Knezl V, Okruhlicová L', Slezák J. Myocardial gap junctions: targets for novel approaches in the prevention of life-threatening cardiac arrhythmias. Physiol Res 2008; 57 (suppl 2): S1 – S13.

36 – Burstein B, Comtois P, Michael G, Nishida K, Villeneuve L, Yeh Y-H, Nattel S. Changes in connexin expression and the atrial fibrillation substrate in congestive heart failure. Circ Res 2009; 105: 1213 – 1222.

37- Chaquor B, Goppelt-Stauebe M. Mechanical regulation of the Cyr61/Ccn1 and Ctgf/Ccn2 proteins. FEBS J 2006; 273 (16): 3639 – 3649.

38- Hill MA, Davis MJ, Meininger GA, Potocnik SJ, Murphy TV. Arteriolar myogenic signaling mechanisms: implications for local vascular function. Clin Hemorheol Microcirc 2006; 34:67-79.

39- Gutstein DE, Liu F, Meyers MB, Choo A, Fishman GI. The organization of adherens junctions and desmosomes at the cardiac intercalated disc is independent of gap junctions. J Cell Sci 2003; 116: 875 – 885.

40- Shimada T, Kawazato H, Yasuda A, Ono N, Sueda K. Cytoarchitecture and intercalated disks of the working myocardium and the conduction system in the mammalian heart. Anat Rec 2004; A280: 940 – 951.

41- Saffitz JE, Hames KY, Kanno S. Remodeling of gap junctions in ischemic and nonischemic forms of heart disease. J Membrane Biol 2007; 218: 65 – 71.

42- Jansen JA, van Veen TAB, Bakker JMT, van Rijen HVM. Cardiac connexins and impulse propagation. Cell Cardiol 2010; 48: 76 – 82.

43- Peters NS, Green CR, Poole-Wilson PA, Severs NJ. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. Circulation 1993; 88: 864 – 875.

44- Kieken F, Mutsaers N, Dolmotova E, Virgil K, Wit AL, Kellezi A, Hirst-Jensen BJ, Duffy HS, Sorgen PL. Structural and molecular mechanisms of gap junction remodeling in epicardial border zone myocytes following myocardial infartion. Circ Res 2009; 104: 1103 – 1112.

45- Gutstein DE, Morley GE Vaidya D, Liu F, Chen FL, Stuhlmann H, Fishman GI. Heterogeneous expression of gap junction channels in the heart leads to conduction defects and ventricular dysfunction. Circulation 2001; 104: 1194 – 2000.

46- Basso C, Czarnowska E, Barbera ML, Bauce B, Beffagna G, Wlodarska EK, Pilichou K, Ramondo A, Lorenzon A, Wozniek O, Corrado D, Daliento L, Danieli GA, Valente M, Nava A, Thiene G, Rampazzo A. Ultrastructural evidence of intercalated disc remodeling in arrhythmogenic right ventricular cardiomyopathy: an electron microscopy investigation on endomyocardial biopsies. Eur Heart J 2006; 27: 1847 – 1854.

47- Pei Z, Sang H, Li R, Xiao P, He J, Zhuang Z, Zhu M, Chen J, Ma H. Infrasound-induced hemodynamics, ultrastructure, and molecular changes in the rat myocardium. Environ Toxicol 2007; 22: 169 – 175.

48- Bishop JE, Lindhal G. Regulation of cardiovascular collagen synthesis by mechanical load. Cardiovasc Res 1999; 42: 27 – 44.

49- Saffitz JE, Kleber AG. Effects of mechanical forces and mediators of hypertrophy on remodeling of gap junction in the heart. Circ Res 2004: 94: 585 – 591.

2. HISTOMORPHOMETRIC EVALUATION OF THE CORONARY ARTERY VESSELS IN RATS SUBMITTED TO INDUSTRIAL NOISE

Eduardo Antunes [(1)], Pedro Oliveira [(1)], Maria João R. Oliveira [(2)], José Brito [(1)], Artur Águas [(2)], José Martins dos Santos [(1)]

(1) *Center for Interdisciplinary Research Egas Moniz, Health Sciences Institute, Monte de Caparica, Portugal*

(2) *Department of Anatomy and UMIB of ICBAS, University of Porto, Porto, Portugal*

Published in Acta Cardiologica 2013; 68: 285 - 289

Abstract

Industrial noise (IN) is characterized by high intensity and a wide spectrum of wavelengths that induce physical vibration on the body structures. This effect resulting from the low-frequency sound waves can lead to pathological alterations in the extracellular matrix with an abnormal proliferation of collagen and development of tissue fibrosis, in the absence of an inflammatory process. **Objective:** The aim of this study was to evaluate the modifications of the arterial coronary vessels in Wistar rats submitted to IN. **Methods:** Two groups of rats were considered: Group A with 20 rats exposed to IN during a maximum period of 7 months; Group B with 20 rats as age-matched controls. The hearts were sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected. Hematoxylin-eosin and Masson's trichrome staining were used for histological observation. Histomorphometric evaluation of the coronary vessels was performed using the computer image analysis *image J software*. The mean lumen-to-vessel wall (L/W) and mean vessel wall-to-perivascular tissue (W/P) ratios were calculated in each group. **Results:** Histological evaluation showed a prominent perivascular tissue with fibrotic development in the absence of inflammatory cells in group A. Histomorphometric analysis showed that the mean L/W was 0.7297 and 0.6940 respectively in group A and B. The mean W/P ratio was 0.4923 and 0.5540 respectively in group A and B being higher in the control group ($p<0.01$). **Conclusions:** There are perivascular structural modifications in arterial coronary vessels. Our results show a significant development of periarterial fibrosis induced by industrial noise in the rat heart.

Key-words: Industrial noise; Coronary histomorphometric analysis; Perivascular fibrosis; Low frequency noise.

Introduction

Industrial noise (IN) is characterized by high intensity and a wide spectrum of wavelengths that induce physical vibration on the body structures (1, 2). This effect resulting from the low-frequency sound waves can lead to pathological alterations characterized by changes in the extracellular matrix with an abnormal proliferation of collagen and development of tissue fibrosis, in the absence of an inflammatory process (3). In the cardiovascular system, morphological changes were observed mainly in blood vessels, cardiac valves and in the pericardium. In blood vessels the effects of low frequency noise result in hyperplasia of the intima and migration of the smooth muscle cells from the media (4). Morphological changes of cardiac valves include thickening, calcification and/or restriction of leaflet movement being the mitral valve the most affected (5). In the pericardium, changes include thickening and additional development of cell layers (6).

Epidemiological studies suggest a relation between noise and hypertension and ischemic heart disease (7, 8). With respect to aircraft noise and hypertension the relative risks range between 1.4 and 2.1 for subjects who live in high exposed areas with an average sound pressure level in the range of 60-70 dB(A). In what concerns ischemic heart disease, prospective studies on road traffic noise showed relative risks ranging from 1.1 to 1.5 for noise levels above 65-70 dB(A).

Results from these studies were only based on sound pressure measured in dB(A), without stratification of the vibration frequency. Nevertheless, these noises are rich in low-frequency components and infrasound, as occurs with the noise of the textile industry.

The aim of this study was to characterize the structural changes in the coronary arterial vessels induced by industrial noise in rats.

Methods

Forty adult Wistar rats from a Spanish breeder (Charles River Laboratories España, SA, Spain) were studied. The animals were treated in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes and with the Portuguese legislation for the same purpose. All the animals were kept in cages, fed standard rat food and had free access to water. Twenty animals (Group A) were exposed to IN for a period of one to seven months, in an occupationally simulated schedule (8 hours/day, 5 days/week, and weekends in silence). The remaining 20 rats were used as age-matched

controls (Group B) and were kept in a silent environment. Each group was divided into four subgroups with five rats and sacrificed after 1, 3, 5, and 7 months.

The sound signal was emitted by an analog noise generator, amplified and frequency-filtered. The noise level was the same as previously reported, characterized by a wide spectrum of frequencies but with an important component under 500 Hz (1).

The hearts were fixed in 10% buffered formalin, sectioned transversely from the ventricular apex to the atria and prepared for histological observation using hematoxylin-eosin and Masson's trichrome staining. The mid-ventricular fragment from each heart was selected for the study.

The histological images were acquired with an optical Leica microscope using 100 x magnification. A total of 130 arterial vessels were selected (61 in group A and 69 in group B). Data were analyzed using the computer image analysis *image J software*. The caliber of the arterial vessels, the thickness of the walls and the perivascular tissue dimension were measured and the mean lumen-to-vessel wall and mean vessel wall-to-perivascular tissue ratios were calculated.

The morphometric data are presented as mean ± SD. In order to assess the effect of exposure to noise and exposure duration on the lumen-to-vessel wall and vessel wall-to-perivascular tissue ratios, a two-way ANOVA model was fit to the data. The appropriateness of the ANOVA model is justified by the fact that the 8 subgroups defined by the factors levels are of equal size (n = 5) which warrants the robustness of the F ratio statistics under potential heterogeneous variances.

Results

The histological evaluation did not show any modifications in the lumen or in the vessel wall, and inflammatory cells were not observed. By contrast, the perivascular tissue was more prominent and seemed to show fibrotic development in IN-exposed rats. Sections of the arterial vessels are shown in figures 1 and 2.

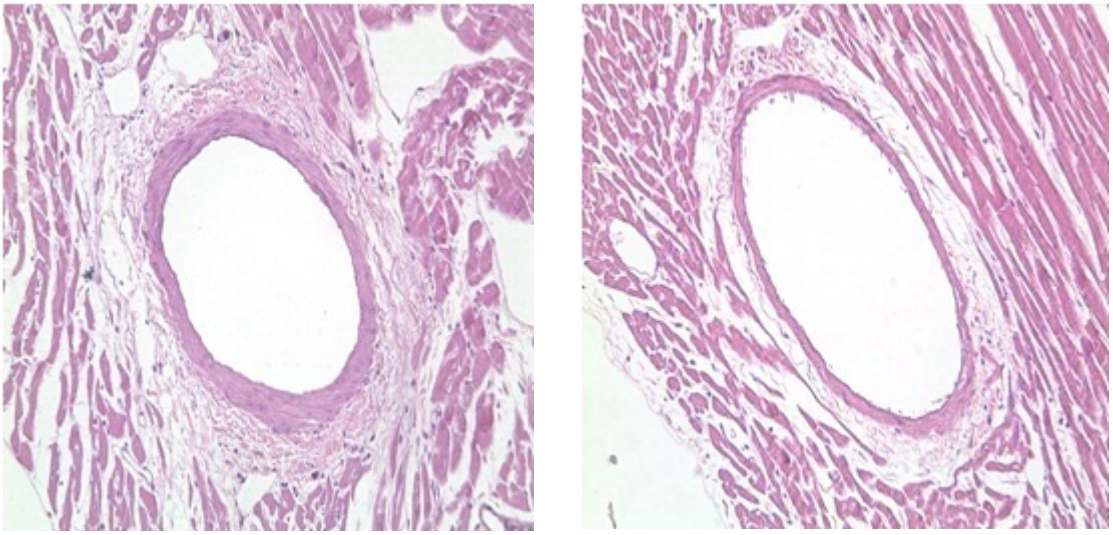
The results of the histomorphometric analysis are shown in table 1, 2 and 3. The mean lumen-to-vessel wall ratio was 0.7297 and 0.6940 respectively in group A and B. The mean vessel wall-to-perivascular tissue ratio was 0.4923 and 0.5540 respectively in group A and B.

The two-way ANOVA analysis of the data of the lumen-to-vessel wall ratio showed that this ratio does not differ significantly between exposed and non-exposed animals ($p = 0.132$) nor does it vary significantly with exposure time ($p = 0.851$). Moreover, no interaction between exposure to noise and duration of exposure has been detected ($p = 0.737$).

Following the two-way ANOVA results, the ratio of vessel wall-to-perivascular tissue differs significantly between exposed and non-exposed animals ($p = 0.009$), with a confidence level of 76.7%, and varies significantly with the exposure duration ($p = 0.012$), with a confidence level of 82.0%. In view of the observed power achieved in the small groups considered in this study, the effects of IN exposure and exposure time are conceivably marked in the whole population. These effects seem to be independent of each other ($p = 0.574$), as suggested in figure 3. The tendency to parallelism showed by the curves demonstrates equal influence of the exposure time on the ratio vessel wall-to-perivascular tissue, in both groups.

Post-hoc tests showed that after a 7-month exposure the ratio vessel wall-to-perivascular tissue is significantly higher than after a 3-month exposure ($p = 0.013$). There is also strong suggestion that the ratio at 7-month exposure is higher than at 1-month exposure although the observed difference does not reach statistical significance ($p = 0.051$).

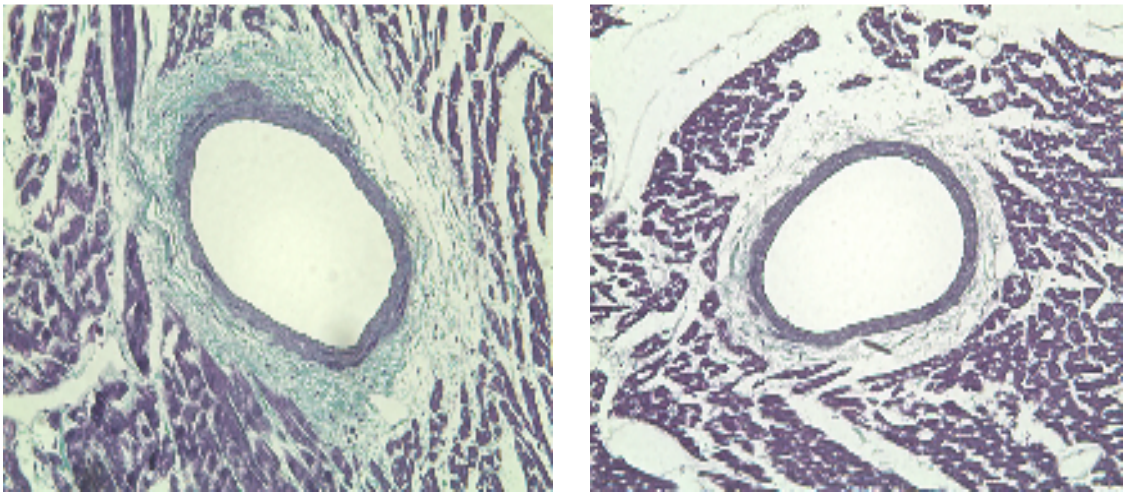
A B



HE x 100

Figure 1 – (A) Coronary artery with a prominent perivascular tissue (IN group). (B) Normal coronary artery (Control group). (Hematoxylin-Eosin x 100).

A B



MT x 100

Figure 2 – (A) Coronary artery with perivascular fibrosis (IN group). (B) Normal coronary artery (control group). (Masson's trichrome x 100).

Table 1- Histomorphometric results of the coronary arteries

	Group A 61 vessels	**Group B 69 vessels**	**p**
Ratio L/W	0.7297 ± 0.0458	0.6940 ± 0.0866	NS
Ratio W/P	0.4923 ± 0.0634	0.5540 ± 0.0905	<0.01

L/W = Mean lumen-to-vessel wall; W/P = Mean vessel wall-to-perivascular tissue

Table 2- Ratio Lumen-to-Vessel Wall

Group	Exposure time (months)	Mean	Std. Deviation	N
IN Exposed (Group A)	1	0,7185	0,0448	5
	3	0,7508	0,0624	5
	5	0,7142	0,0288	5
	7	0,7354	0,0467	5
	Total	**0,7297**	**0,0458**	**20**
Control (Group B)	1	0,6902	0,0367	5
	3	0,6709	0,1034	5
	5	0,6923	0,1341	5
	7	0,7226	0,0627	5
	Total	**0,6940**	**0,0866**	**20**

Table 3 - Ratio Vessel Wall-to-Perivascular Tissue

Group	Exposure time (months)	Mean	Std. Deviation	N
IN Exposed (Group A)	1	0,4687	0,0689	5
	3	0,4582	0,0166	5
	5	0,4807	0,0561	5
	7	0,5617	0,0509	5
	Total	**0,4923**	**0,0634**	**20**
Control (Group B)	1	0,5231	0,0931	5
	3	0,4968	0,0733	5
	5	0,5959	0,0978	5
	7	0,6000	0,0721	5
	Total	**0,5540**	**0,0905**	**20**

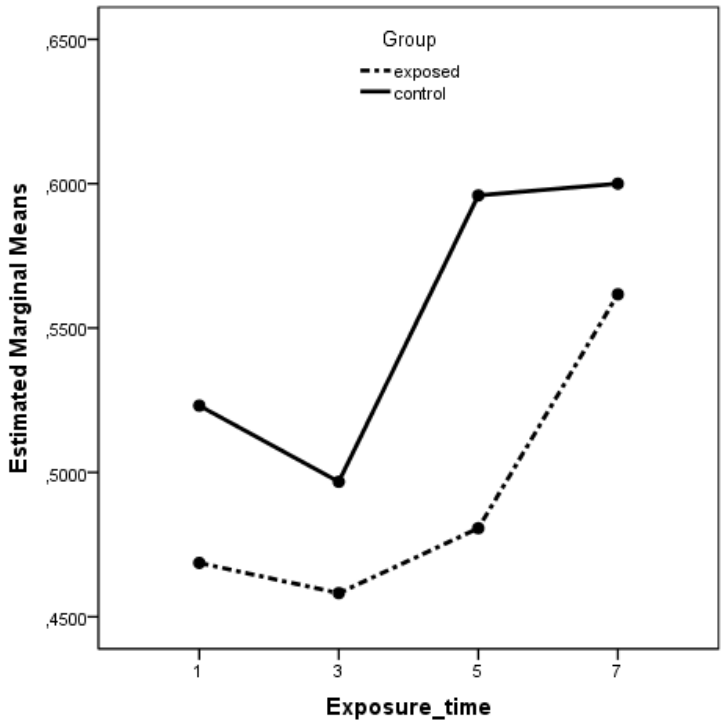


Figure 3 – Estimated marginal means of the ratio vessel wall-to-perivascular tissue

Discussion

As far as we know this is the first study concerning the histomorphometric evaluation of the coronary arterial vessels in rats submitted to industrial noise. Previous studies showed modifications in several tissues induced by low frequency noise, characterized by abnormal deposition of collagen in the extracellular matrices (6, 9, 10).

In contrast to previous studies performed on large vessels (4), modifications in the lumen and vessel wall were not observed. In this study, neither disruption of the internal elastic lamina nor proliferation of smooth muscle cells in the intima were observed, suggesting a lower susceptibility of the coronary arteries to IN damage. Also, the histomorphometric analysis showed no significant differences concerning the ratio lumen-to-vessel wall between the two groups.

On the other hand, our results showed an increase of the perivascular tissue in rats exposed to IN including the development of perivascular fibrosis, in the absence of inflammatory cells and in the absence of coronary artery disease. The statistical evaluation detected significant differences between the groups concerning the mean vessel wall-to-perivascular tissue ratio, being higher among control rats ($p = 0.009$). The effects of exposure time were observed in the whole population of rats and seemed to be independent of exposure to IN. At 3 months of IN exposure, the perivascular tissue was more exuberant and though a reduction was observed at 7 months, it remained increased among the IN-exposed rats.

In the heart, fibroblasts are critical cells not only to normal myocardial function but also in the remodeling that occurs in response to pathological changes such as hypertension, myocardial infarction and heart failure. Many of the effects are mediated through the differentiation into myofibroblasts which exhibit proliferative, migratory and secretory properties and are responsive to proinflammatory cytokines, growth factors and vasoactive peptides such as angiotensin II (11-14).

Collagen produced by these cells has a crucial role in maintaining the myocardial structure and cardiac function, because of its contact proximity to cell and non-cell components of the myocardium. The extracellular matrix homeostasis requires a balance between the synthesis and degradation of collagen and changes in this interaction result in an abnormal collagen network in the heart.

Myocardial fibrosis reflects an excess of collagen in the extracellular matrix and has important clinical implications. In myocardial infarction its presence in locations remote to the necrotic area contributes to the development of cardiac remodeling and ventricular

dysfunction (13). In hypertensive heart disease, myocardial fibrosis has an interstitial and perivascular location being the result of an exaggerated synthesis of collagen I and III and a reduced activity of the metalloproteinases (14). In hypertrophic cardiomyopathy the left ventricular myocardial fibrosis occurs in a large proportion of patients and seems to be related with an adverse prognosis (15). In idiopathic dilated cardiomyopathy, an increase of interstitial fibrosis is also associated with a worse prognosis and different response to treatments (16). In elderly patients, increased collagen types I and III turnover, assessed by elevated fibrosis markers, may reflect diastolic or systolic heart failure (17). In addition, the occurrence of myocardial fibrosis can lead to regional electrophysiological heterogeneities that contribute to the genesis of atrial and ventricular arrhythmias (18-20). From all these studies one can extrapolate that the development of myocardial and perivascular fibrosis can modify the prognosis of several cardiomyopathies through the deterioration of systolic and diastolic ventricular function and by creating an arrhythmic substrate.

Taking into consideration that perivascular fibrosis is also found in hypertensive heart disease, one can speculate that IN can induce fibrosis through the loss of regulation between profibrotic and antifibrotic molecules, carried out by mechanical and neuro-humoral factors (14). From a theoretically point of view and taking into account the dynamic interactions between fibroblasts and the extracellular matrix (21), another possibility could be the occurrence of an abnormal biological fibroblastic response induced by IN through a mecanotransduction process (3).

Regardless of the perivascular fibrotic proliferation mechanism, it will be important to define what is the pathological cardiac model induced by IN in order to better predict what could be the clinical impact on cardiac diseases among people exposed to high intensity/low frequency noise. Additionally it will be a challenge to understand whether people with normal hearts are also prone to develop coronary perivascular fibrosis.

In conclusion: There are perivascular structural modifications in arterial coronary vessels. Our results show a significant development of periarterial fibrosis induced by industrial noise in the rat heart.

Footnotes

Conflict of interest: none declared

References

1- Oliveira MJR, Pereira AS, Ferreira PG, Guimarães L, Freitas D, Carvalho APO, Grande NR, Águas AP. Arrest in ciliated cell expansion on the bronchial lining of adults rats caused by chronic exposure to industrial noise. Environmental Research 2005; 97: 282 – 286.

2- Oliveira MJR, Monteiro M, Ribeiro AM, Pignatelli D, Águas AP. Chronic exposure of rats to occupational textile noise cause cytological changes in adrenal cortex. Noise Health 2009; 11(43): 118 – 123.

3- Alves-Pereira M, Castelo Branco NAA. Vibroacoustic disease: biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signaling. Prog Bioph Mol Biol 2007; 93: 256 – 279.

4- Martins dos Santos J, Grande NR, Castelo Branco NA, Zagalo C, Oliveira P. Vascular lesions and vibroacoustic disease. Eur J Anat 2002; 6 (1): 17 – 21.

5- Marciniak W, Rodriguez E, Olszowska K, Atkov O, Botvin I, Araujo A, Pais F, Ribeiro CS, Bordalo A, Prazeres de Sá E, Ferreira D, Loureiro J, Castelo Branco MSN, Castelo Branco NAA. Echocardiographic evaluation in 485 aeronautical workers exposed to different noise environments. Aviat Space Environ Med 1999; 70 (3 suppl): A46 – A53.

6- Castelo Branco NAA, Águas AP, Pereira AS, Monteiro E, Fragata JIG, Tavares F, Grande NR. The human pericardium in vibroacoustic disease. Aviat Space Environ Med 1999; 70 (3 suppl): A54 – A62.

7- Babish W. Road traffic noise and cardiovascular risk. Noise & Health 2009; 10: 27 – 33.

8- Babish W. Transportation noise and cardiovascular risk: updated review and synthesis of epidemiological studies indicate that the evidence has increased. Noise & Health 2009; 8: 1 – 29.

9- da Fonseca J, dos Santos JM, Branco NC, Alves-Pereira M, Grande NR, Oliveira P, Martins AP. Noise-induced gastric lesions: a light and scanning electron microscopy study of the alterations of the rat gastric mucosa induced by low frequency noise. Cent Eur J Public Health 2006; 14 (1): 35 – 38.

10- Grande NR, Águas AP, Sousa Pereira A, Monteiro E, Castelo Branco NAA. Morphological changes in the rat lung parenchyma exposed to low frequency noise. Aviat Space Environ Med 1999; 70: A70 - A77.

11- Brilla CG, Scheer C, Rupp H. Renin-angiotensin system and myocardial collagen matrix: Modulation of cardiac fibroblast function by angiotensin II type 1 receptor antagonism. J Hypertens 1997; 15 (Suppl 6): S13 – S19.

12- Petrov VV, Fagard RH, Lijnen PJ. Stimulation of collagen production by transforming growth factor-β1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 2002; 39:258 – 263.

13- van den Borne SWM, Diez J, Blankesteijn M, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 2010; 7: 30 – 37.

14- Diez J. Mechanisms of cardiac fibrosis in hypertension. J Clin Hypertens 2007; 9: 546 – 550.

15 – Pujadas S, Carreras F, Arrastio X, Leta R, Vila M, Subirana MT, Bayés-Genis A, Pons-Lladó G. Detection and quantification of myocardial fibrosis in hipertrophic cardiomyopathy by contrast enhanced cardiovascular magnetic resonance. Rev Esp Cardiol 2007; 60 (1): 10 – 14.

16- Bruckner BA, Razeghi P, Stetson S, Thompson L, Lafuente J, Entman M, Loebe M, Noon G, Taegtmeyer H, Frazier OH, Youker K. Degree of cardiac fibrosis and hypertrophy at time of implantation predicts myocardial improvement during left ventricular assist device support. J Heart Lung Transplant 2004; 23 (1): 36 – 42.

17- Barasch E, Gottdiener JS, Aurigemma G, Kitzman DW, Han J, Kop WJ, Tracy RP. Association between elevated fibrosis markers and heart failure in the elderly.The cardiovascular health study. Circ Heart Fail 2009; 2: 303 – 310.

18- Tribulová N, Knezl V, Okruhlicová L', Slezák J. Myocardial gap junctions: targets for novel approaches in the prevention of life-threatening cardiac arrhythmias. Physiol Res 2008; 57 (suppl 2): S1 – S13.

19- Burstein B, Comtois P, Michael G, Nishida K, Villeneuve L, Yeh Y-H, Nattel S. Changes in connexin expression and the atrial fibrillation substrate in congestive heart failure. Circ Res 2009; 105: 1213 – 1222.

20- Iv THE, Olgin JE. Atrial fibrosis and the mechanisms of atrial fibrillation. Heart Rhythm 2007; 4 (3 suppl): S24 – S27.

21- Banerjee I, Yekkala K, Borg TK, Baudino TA. Dynamic interactions between myocytes, fibroblasts, and extracellular matrix. Ann N Y Acad Sci 2006; 1080: 76 – 84.

3. MYOCARDIAL FIBROSIS IN RATS EXPOSED TO LOW-FREQUENCY NOISE

Eduardo Antunes [(1)], Pedro Oliveira [(1)], Gonçalo Borrecho [(1)], Maria João R. Oliveira [(2)], José Brito [(1)], Artur Águas [(2)], José Martins dos Santos [(1)]

(1) Center for Interdisciplinary Research Egas Moniz, Health Sciences Institute, Monte de Caparica, Portugal

(2) Department of Anatomy and UMIB of ICBAS, University of Porto, Porto, Portugal.

Published in Acta Cardiologica 2013; 68: 241 - 245

Abstract

Low frequency noise (LFN) characterized by large pressure amplitude (≥ 90 dB SPL) and low frequency bands (≤ 500 Hz) can lead to structural and ultrastructural modifications in the extracellular matrix of several tissues, with an abnormal proliferation of collagen and development of fibrosis. It is not known whether LFN induces similar structural alterations in the ventricular myocardium of rats. **Objective:** The aim of this study was to evaluate and measure the myocardial fibrosis induced by LFN. **Methods:** Two groups of rats were considered: Group A with 26 rats continuously exposed to LFN during a period of 3 months; Group B with 20 control rats. The hearts were sectioned from the ventricular apex to the atria and the medium-ventricular fragment was selected. Chromotrope-aniline blue (CAB) staining was used for histological observation. The measurement of fibrosis was performed using the computer image analysis *image J software.* **Results:** Histological observation with CAB staining showed the presence of collagen deposition between the cardiomyocytes. Fibrosis increased 97.5%, 81.5% and 83.7%, respectively in the left ventricle, interventricular septum and right ventricle, in exposed rats ($p<0.001$). The ratio fibrosis/muscle in left ventricle, interventricular septum and right ventricle was significantly higher in LFN exposed rats ($p<0.001$). **Conclusions:** Our study demonstrates a significant myocardial fibrosis induced by low frequency noise in rats. Our results reinforce the need for further experimental and clinical investigations concerning the effects of low frequency noise on the heart.

Key-words: Low frequency noise; chromotrope-aniline blue staining; ventricular myocardial fibrosis.

Introduction

Low frequency noise (LFN) characterized by large pressure amplitude (≥ 90 dB SPL) and low frequency bands (≤ 500 Hz) can lead to structural and ultrastructural modifications in the extracellular matrix of several organs, with an abnormal proliferation of collagen and development of tissue fibrosis (1). In animal models, fibrosis was already found in lung parenchyma, tracheal epithelia, gastric mucosa, lymphatic vessels, arterial vessels and parotid gland after LFN exposure (2 - 7).

The importance of looking into the effects of LFN on the heart is the increased evidence relating noise and hypertension and ischemic heart disease (8). In this mentioned report, a review of epidemiological studies in the field of community noise and cardiovascular risk was made, considering the road and aircraft noise sources which are rich in low-frequency components and infrasound.

Additionally, taking into consideration the effects of LFN on the extracellular matrix of several tissues, one can admit the occurrence of myocardial fibrosis in LFN-exposed rats. The clinical consequences would be inherent to the deposition of collagen between the cardiomyocytes and around the cardiac vessels leading to myocardial stiffness and left ventricular dysfunction. These pathological modifications could give rise to the occurrence of cardiac heart failure and arrhythmias (9, 10).

In the heart, LFN-induced fibrotic development was observed mainly in cardiac valves and pericardium (11, 12), but it is not known whether LFN induces similar structural alterations in the rat ventricular myocardium.

Thus, we hypothesized that LFN can induce cardiac morphological alterations not just confined to the pericardium and valves but also on the myocardium and this study was undertaken to evaluate and measure the myocardial fibrosis induced by LFN.

Methods

Forty-six adult Wistar rats from a Spanish breeder (Charles River Laboratories España, SA, Spain) were studied. The animals were treated in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes and with the Portuguese legislation for the same purpose. Twenty-six rats (Group A) were continuously exposed to LFN for a period of three months. The control group of 20 rats (Group B) was kept in a silent environment. All the animals were kept in cages, fed standard rat food and had free access to water.

The sound signal was emitted by an analog noise generator and the noise level was the same as previously reported (13).

The hearts were fixed in 10% buffered formalin, transversely sectioned into four parts from the ventricular apex to the atria and prepared for histological observation using chromotrope-aniline blue (CAB) staining. The medium ventricular fragment from each heart was selected for the study.

The histological images were acquired with an optical Leica microscope using 100 x magnification. In each section one field was selected from the left ventricle, interventricular septum and right ventricle. In order to exclude the presence of perivascular collagen, criteria used to select each field were defined by the myocardium portion with more fibrotic development in the absence of any arterial vessel.

A total of 138 optical fields were selected by three observers, under blinded assessment, and analyzed using the *Image J software* that gives a quantification based on the image colour analysis. In each field an ideal cutoff was defined as the strongest correlation between the histological observation and the digital colour gradients evaluation. This ideal cutoff was used to quantify the presence of collagen and cardiac muscle and the following parameters were measured: 1- Cardiac muscle 2- Interstitial fibrosis. A ratio of fibrosis/muscle was then calculated.

Data are presented as mean ± SD. Comparisons among groups and anatomical regions were performed by repeated measures ANOVA and MANOVA one-way. A value of $p<0.05$ was considered statistically significant.

Results

The histological observation showed collagen deposition between the cardiomyocytes predominantly in rats exposed to LFN. CAB staining samples from group A and B are shown in Fig. 1.

As expected, the mean values of cardiac muscle were significantly different between the right ventricle and left ventricle ($p<0.001$), and between the right ventricle and the interventricular septum ($p<0.001$) being lower in the right ventricle but there were no differences between the exposed and non-exposed rats (Fig. 2).

The mean values of interstitial fibrosis in left ventricle, interventricular septum and right ventricle are illustrated in table 1 and Fig. 3. The results were significantly higher in group

A ($p<0.001$). Fibrosis increased in the exposed rats by 97.5%, 81.5% and 83.7%, respectively in the left ventricle, interventricular septum and right ventricle ($p < 0.001$).

The ratio fibrosis/muscle did not show significant differences between the left ventricle, interventricular septum and right ventricle (Table 2 and Fig. 4). However the ratio fibrosis/muscle was significantly higher in the LFN exposed rats ($p<0.001$).

Table 1- Interstitial Fibrosis

	Group A (26 LFN exposed rats)	Group B (20 control rats)	Percent increase with LFN exposure	p
LV	**40.6 ± 15.6**	**20.5 ± 7.2**	**97.5%**	**<0.001**
IS	**37.5 ± 17.2**	**20.6 ± 8.0**	**81.5%**	**<0.001**
RV	**35.0 ± 14.4**	**19.1 ± 8.2**	**83.7%**	**<0.001**

LV = Left Ventricle; IS = Interventricular Septum; RV = Right Ventricle; LFN = Low Frequency Noise

Table 2- Ratio Fibrosis/Muscle

	Group A (26 LFN exposed rats)	Group B (20 control rats)	p
LV	**0.176 ± 0.072**	**0.091 ± 0.034**	**<0.001**
IS	**0.170 ± 0.078**	**0.090 ± 0.039**	**<0.001**
RV	**0.182 ± 0.077**	**0.108 ± 0.036**	**<0.001**

LV = Left Ventricle; IS = Interventricular Septum; RV = Right Ventricle; LFN = Low Frequency Noise

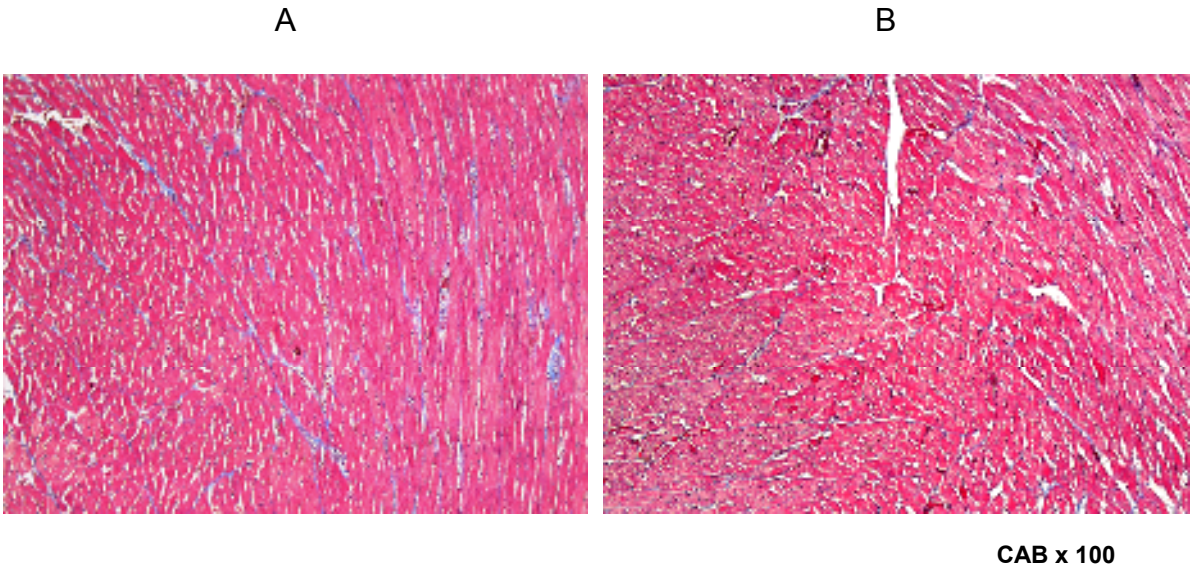


Figure 1 – Interstitial fibrosis observed between the cardiomyocytes in LFN group (A) and in control group (B). (CAB staining x 100).

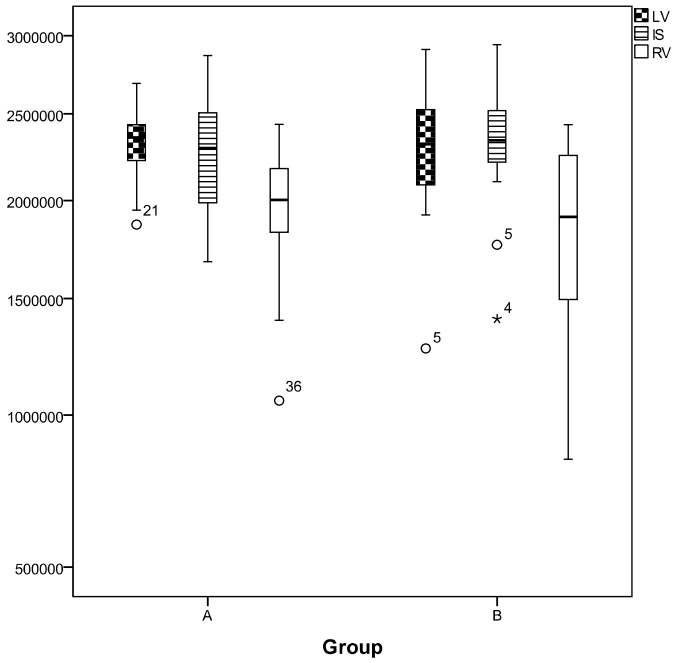


Figure 2 – Quantification of cardiac muscle in the left ventricle (LV), interventricular septum (IS) and right ventricle (RV) in animals exposed to low frequency noise (group A) and in controls (group B). In each group the quantity of muscle was lower in the right ventricle but there were no significant differences between the two groups.

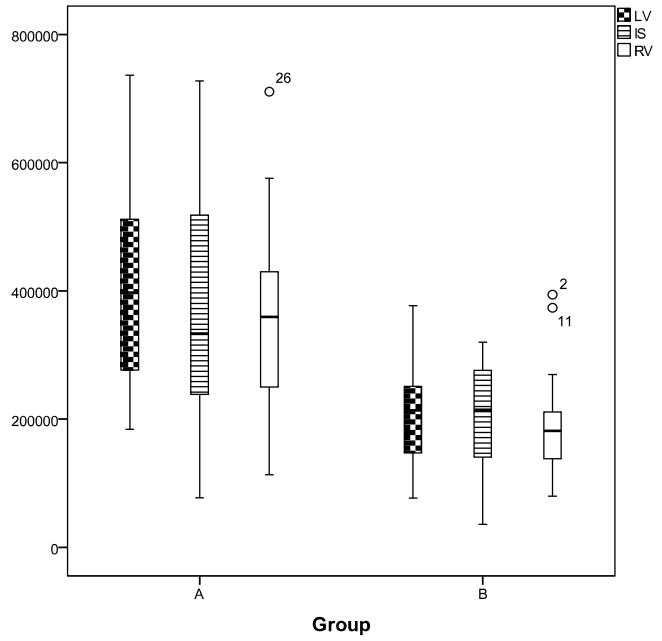


Figure 3 - Quantification of interstitial fibrosis in the left ventricle (LV), interventricular septum (IS) and right ventricle (RV) in animals exposed to low frequency noise (group A) and in controls (group B). In each group there are no significant differences among the anatomical regions but a significant increase of myocardial fibrosis is observed among the rats exposed to low frequency noise ($p < 0.001$).

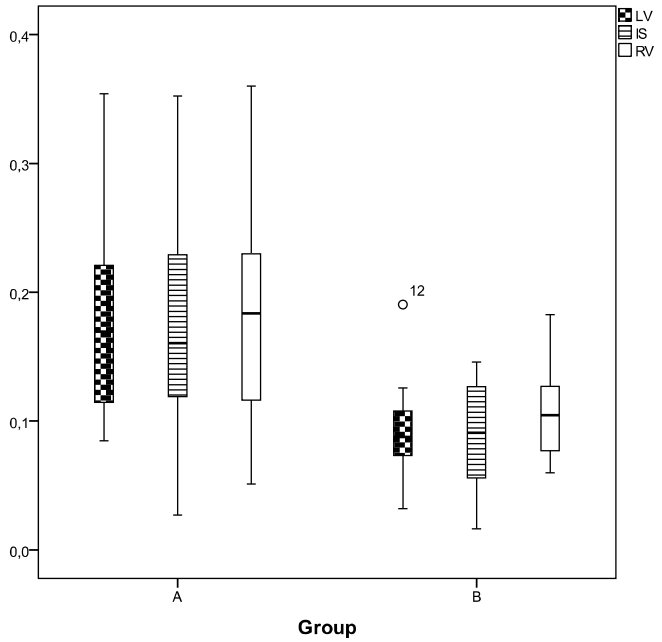


Figure 4 – Ratio fibrosis/muscle in the left ventricle (LV), interventricular septum (IS) and right ventricle (RV) in animals exposed to low frequency noise (group A) and in controls (group B). A significant increase is observed in rats exposed to low frequency noise ($p < 0.001$).

Discussion

To the best of our knowledge this is the first study showing fibrotic development of the ventricular myocardium in rats exposed to LFN. Previous studies reported modifications in several tissues induced by LFN, characterized by abnormal deposition of collagen in the extracellular matrix (2 - 7). In the heart, morphological alterations were observed in cardiac valves and in the pericardium, but it is not known whether LFN induces fibrosis in the ventricular myocardium (11, 12).

In this study, a marked development of interstitial fibrosis was observed in both ventricles and interventricular septum without important differences between anatomical regions, suggesting a general myocardial fibroblastic response induced by LFN. This abnormal proliferation of connective tissue has been the main consequence induced by LFN in other biological tissues. The cause of the fibrotic proliferation mechanism is still unknown but it possibly could be an abnormal fibroblastic response induced by LFN through a mecanotransduction process (1). The loss of regulation between profibrotic and antifibrotic molecules, linked to mechanical and neuro-humoral factors, as occurs in hypertension, could be another mechanism (14).

In the heart, fibroblasts are critical cells in the remodeling that occurs in response to pathological changes such as, hypertension, myocardial infarction and heart failure (14, 17). In some diseases as hypertension or conditions with hemodynamic overload, fibroblasts are under a mechanical stress, and exhibit proliferative properties (18, 19). From a biophysical point of view, one can admit that the mechanical impact of LFN can have the same effects. In fact, *in vitro* studies showed histomorphometric, phenotypical and functional significant changes when mechanical forces were applied to cells (20 - 22).

Whatever the fibrotic proliferation mechanism, myocardial ventricular fibrosis reflects an excess of collagen in the extracellular matrix and can have important clinical implications (14, 17, 23, 24). These clinical consequences are mainly related to ventricular dysfunction, modifications in coronary flow reserve and ventricular arrhythmias (9, 10, 25, 26). Fibrosis induces stiffness of the myocardial tissue (9) and it was already showed that perivascular fibrosis can limit vessel distensibility (26). Fibrosis can also induce electrophysiological heterogeneities leading to ventricular arrhythmias (10).

Some clinical descriptions of myocardial fibrosis are inherent to specific cardiac diseases as hypertensive, ischemic or hypertrophic cardiomyopathies (14, 17, 23, 24), which makes clinically difficult to predict similar structural changes induced by LFN in these patients. Nevertheless, as fibrosis induces ventricular wall rigidity it will be important to evaluate the

ventricular diastolic function among individuals exposed to LFN. In this regard, a recent study showed modifications on the echocardiographic parameter E/A ratio suggesting that it could be a feasible and fast marker for screening the effects of LFN on the heart (27).

Meanwhile, it is fundamental to define the morphological cardiac model induced by LFN in order to better understand the clinical consequences in exposed people. This pathological model should be based on vascular, myocardial and electrophysiological components whose alterations have unquestionable clinical implications. Our study was limited to the observation of structural modifications in the myocardium. The electrophysiological and vascular components were beyond the scope of this study. It will be also important to evaluate the type of collagen responsible for the reported fibrosis, as serum markers of fibrosis, which reflect myocardial collagen turnover, can be used in clinics (25, 28, 29).

It is expected that the clinical impact of any morphological tissue modification induced by LFN will be more accentuated in LFN-exposed professions such as commercial and military pilots, cabin crewmembers, aircraft technicians, ship machinists (30). Also, workers of textile industrial plants are exposed to high density/low-frequency noise and have increased respiratory infections and modifications in respiratory function (31, 32). In rats exposed to the same noise spectrum using a similar simulated schedule, an impairment of ciliated cell expansion on the bronchial epithelium was observed (33) as well as cytological changes in the adrenal cortex (34).

In a study conducted in aircraft technicians, three clinical stages with progressively severe pathologies were defined, according to 2, 5 or more than 10 years of LFN-exposure (35). Cardiac alterations such as thickening of the valves and pericardium were reported but the relation with the LFN-exposure was not established.

Our study was in animals and it is always difficult to extrapolate to humans, anyhow the fibrotic myocardial development was observed after a 3-month continuous LFN exposure which is similar to 12 months in a schedule characterized by 8 hours/day, 5 days/week, as occurs with industrial plant workers.

In conclusion, our study demonstrates a significant ventricular myocardial fibrosis induced by low frequency noise, in rats. Our results reinforce the need for further experimental and clinical investigations concerning the effects of low frequency noise on the heart.

Footnotes

Conflict of interest: none declared

References

1- Alves-Pereira M, Castelo Branco NAA. Vibroacoustic disease: biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signaling. Prog Bioph Mol Biol 2007; 93: 256 – 279.

2- da Fonseca J, dos Santos JM, Branco NC, Alves-Pereira M, Grande NR, Oliveira P, Martins AP. Noise-induced gastric lesions: a light and scanning electron microscopy study of the alterations of the rat gastric mucosa induced by low frequency noise. Cent Eur J Public Health 2006; 14 (1): 35 – 38.

3- Grande NR, Águas AP, Sousa Pereira A, Monteiro E, Castelo Branco NAA. Morphological changes in the rat lung parenchyma exposed to low frequency noise. Aviat Space Environ Med 1999; 70: A70 – A77.

4- de Sousa Pereira A, Águas AP, Grande NR, Mirones J, Monteiro E, Castelo Branco NAA. The effect of chronic exposure to low frequency noise on rat tracheal epithelia. Aviat Space Environ Med. 1999; 70: A186 – A190.

5- Martins dos Santos J, Grande NR, Castelo Branco NAA, Zagalo C, Oliveira P, Alves-Pereira M. Lymphatic lesions ond vibroacoustic disease. Eur J Lymphol 2004; 12: 17 – 20.

6- Oliveira PM, Pereira da Mata AD, Martins dos Santos JA, da Silva Marques DN, Branco NC, Silveira JM, Correia da Fonseca JC. Low-frequency noise effects on the parotid gland of the Wistar rat. Oral Dis 2007; 13 (5): 468 – 473.

7- Martins dos Santos J, Grande NR, Castelo Branco NA, Zagalo C, Oliveira P. Vascular lesions and vibroacoustic disease. Eur J Anat 2002; 6 (1): 17 – 21.

8- Babish W. Transportation noise and cardiovascular risk: updated review and synthesis of epidemiological studies indicate that the evidence has increased. Noise Health 2009; 8: 1 – 29.

9- Weber KT, Brilla CG, Janicki JS. Myocardial fibrosis. Functional significance and regulatory factors. Cardiovasc Res 1993; 27: 13 – 31.

10- Assyang P, Carré F, Chevalier B, Delcayre C, Mansier P, Swing-hedouw B. Compensated cardiac hypertrophy: arrhythmogenicity and the new myocardial phenotype. I. Fibrosis. Cardiovasc Res 1997; 34: 439 – 444.

11- Marciniak W, Rodriguez E, Olszowska K, Atkov O, Botvin I, Araujo A, Pais F, Ribeiro CS, Bordalo A, Prazeres de Sá E, Ferreira D, Loureiro J, Castelo Branco MSN, Castelo

Branco NAA. Echocardiographic evaluation in 485 aeronautical workers exposed to different noise environments. Aviat Space Environ Med 1999; 70 (3 suppl): A46 – A53.

12- Castelo Branco NAA, Águas AP, Pereira AS, Monteiro E, Fragata JIG, Tavares F, Grande NR.The human pericardium in vibroacoustic disease. Aviat Space Environ Med 1999; 70 (3 suppl): A54 – A62.

13- da Fonseca J, Martins dos Santos, J,Castelo-Branco N, Alves-Pereira M, Grande N, Oliveira P. Noise-induced duodenal lesions. Eur J Anat 2005; 9 (1): 29 – 33.

14- Diez J. Mechanisms of cardiac fibrosis in hypertension. J Clin Hypertens 2007; 9: 546 – 550.

15- Brilla CG, Scheer C, Rupp H. Renin-angiotensin system and myocardial collagen matrix: modulation of cardiac fibroblast function by angiotensin II type 1 receptor antagonism. J Hypertens 1997; 15 (Suppl 6): S13 – S19.

16- Petrov VV, Fagard RH, Lijnen PJ. Stimulation of collagen production by transforming growth factor-β1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 2002; 39: 258 – 263.

17- van den Borne SWM, Diez J, Blankesteijn M, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 2010; 7: 30 – 37.

18- Chaquor B, Goppelt-Stauebe M. Mechanical regulation of the Cyr61/Ccn1 and Ctgf/Ccn2 proteins. FEBS J 2006; 273 (16): 3639 – 49.

19- Hill MA, Davis MJ, Meininger GA, Potocnik SJ, Murphy TV. Arteriolar myogenic signaling mechanisms: implications for local vascular function. Clin Hemorheol Microcirc 2006; 34: 67 – 79.

20- Ingber DE. Mechanical signaling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. Circ Res 2002; 91: 877-887.

21- Silver FH, Devore D, Siperko LM. Role of mechanophysiology in aging of ecm: effects of changes in mechanochemical transduction. J Appl Physiol 2003; 95: 2134-2141.

22- Janmey PA, Weitz DA. Dealing with mechanics: mechanisms of force transduction in cells. Trends Biochem Sci 2004; 29: 364-370.

23- Pujadas S, Carreras F, Arrastio X, Leta R, Vila M, Subirana MT, Bayés-Genis A, Pons-Lladó G. Detection and quantification of myocardial fibrosis in hipertrophic cardiomyopathy by contrast enhanced cardiovascular magnetic resonance. Rev Esp Cardiol 2007; 60 (1): 10 – 14.

24- Bruckner BA, Razeghi P, Stetson S, Thompson L, Lafuente J, Entman M, Loebe M, Noon G, Taegtmeyer H, Frazier OH, Youker K. Degree of cardiac fibrosis and hypertrophy at time of implantation predicts myocardial improvement during left ventricular assist device support. J Heart Lung Transplant 2004; 23 (1): 36 - 42.

25- Barasch E, Gottdiener JS, Aurigemma G, Kitzman DW, Han J, Kop WJ, Tracy RP. Association between elevated fibrosis markers and heart failure in the elderly. The cardiovascular health study. Circ Heart Fail 2009; 2: 303 – 310.

26- Isoyama S, Ito N, Satoh U, Takishima T. Collagen deposition and the reversal of coronary reserve in cardiac hypertrophy. Hypertension1992; 20: 491 - 500.

27- Chao P-C, Yeh C-Y, Juang Y-J, Hu C-Y, Chen C-J. Effect of low frequency noise on the echocardiographic parameter E/A ratio. Noise Health 2012; 14 (59): 155 – 158.

28- Izawa H, Murohara T, Nagata K, Isobe S, Asano H, Amano T, Ichihara S, Kato T, Ohshima S, Murase Y, Iino S, Obata K, , Noda A, Okumura K, Yokota M. Mineralocorticoid recepter antagonism ameliorates left ventricular diastolic dysfunction and myocardial fibrosis in mildly symptomatic patients with idiopatic dilated cardiomyopathy: a pilot study. Circulation 2005; 112: 2940 – 2945.

29- Schwartzkopff B, Fassbach M, Pelzer B, Brehm M, Strauer BE. Elevated serum markers of collagen degradation in patients with mild to moderate dilated cardiomyopathy. Eur J Heart Fail 2002; 4: 434 - 439.

30- Castelo-Branco NAA, Alves-Pereira M. Vibroacoustic disease. Noise Health 2004; 6 (23): 3 – 20.

31- Fishwick D, Fletcher AM, Pickering CA, McL Niven R, Faragher EB. Lung function in Lancashire cotton and man made fibre spinning mill operatives. Occup Environ Med 1996; 53 (1): 46 – 50.

32- Cardoso AP, Oliveira MJ, Silva AM, Águas AP, Pereira AS. Effects of long term exposure to occupational noise on textile industry workers' lung function. Rev Port Pneumol 2006; 12: 45 – 59.

33- Oliveira MJR, Pereira AS, Ferreira PG, Guimarães L, Freitas D, Carvalho APO, Grande NR, Águas AP. Arrest in ciliated cell expansion on the bronchial lining of adults rats caused by chronic exposure to industrial noise. Environmental Research 2005; 97: 282 – 286.

34- Oliveira MJR, Monteiro M, Ribeiro AM, Pignatelli D, Águas AP. Chronic exposure of rats to occupational textile noise cause cytological changes in adrenal cortex. Noise Health 2009; 11(43): 118 – 123.

35- Castelo-Branco NAA. The clinical stages of vibroacoustic disease. Aviat Space Environ Med 1999; 70 (3 suppl): A32 – A39.

4. IMMUNOHISTOCHEMICAL EVALUATION OF CARDIAC CONNEXIN43 IN RATS EXPOSED TO LOW-FREQUENCY NOISE

Eduardo Antunes [(1)], Gonçalo Borrecho [(1)], Pedro Oliveira [(1)], José Brito [(1)], Artur Águas [(2)], José Martins dos Santos [(1)]

(1) *Center for Interdisciplinary Research Egas Moniz, Health Sciences Institute, Monte de Caparica, Portugal*

(2) *Department of Anatomy and UMIB of ICBAS, Abel Salazar Institute for Biomedical Sciences, University of Porto, Porto, Portugal*

Published in the International Journal of Clinical and Experimental Pathology 2013; 6: 1874 -1879

Abstract

Introduction: Low-frequency noise (LFN) leads to an abnormal proliferation of collagen and development of tissue fibrosis. It has been shown that myocardial fibrosis in association with gap junction remodeling occurs in several cardiac diseases and can be implicated in the development of ventricular tachyarrhythmias. We previously reported a strong development of myocardial fibrosis induced by LFN in rats but it is not known whether LFN induces any modification on cardiac connexin43 (Cx43). **Objectives**: The aim of this study was to evaluate modifications on cardiac Cx43 induced by LFN in Wistar rats. **Methods:** Two groups of rats were considered: A LFN-exposed group with 10 rats submitted continuously to LFN during 3 months and a control group with 8 rats. The hearts were sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected. The immunohistochemical evaluation of Cx43 was performed using the polyclonal antibody connexin-43m diluted 1:1000 overnight at 4º C. Quantifications of Cx43 and muscle were performed with the *image J software* and the ratio Cx43/muscle was analyzed in the left ventricle, interventricular septum and right ventricle. **Results:** The ratio Cx43/muscle was significantly reduced in LFN-exposed rats (p=0.001). The mean value decreased 46.2%, 22.2% and 55.6% respectively in the left ventricle (p=0.008), interventricular septum (p=0.301) and right ventricle (p=0.004). **Conclusions:** LFN induces modifications on cardiac Cx43 in rats. The Cx43 reduction observed in our study suggests that LFN may induce an arrhythmogenic substrate and opens a new investigational area concerning the effects of LFN on the heart.

Key-words: Low-frequency noise; Connexin43; Gap junction; Intercalated disks; Ventricular myocardium.

Introduction

Low-frequency noise (LFN) leads to pathological changes in the extracellular matrix, characterized by an abnormal proliferation of collagen and the development of tissue fibrosis, in the absence of an inflammatory process (1-7). We previously reported a significant fibrotic development in ventricular myocardium of rats exposed to LFN (8) and an increase of perivascular fibrosis in the arterial coronary vessels after exposure to industrial noise which is rich in LFN components (9), but it is not known whether LFN induces modifications on the electrophysiological *milieu*.

Gap junctions are composed by proteins known as connexins, form the intercellular pathway for electrical impulse transmission and are determinants in the genesis of cardiac arrhythmias. Changes on gap junctional connexin43 (Cx43) have been implicated in ventricular remodeling and development of arrhythmias in several cardiac diseases (10-16). Additionally, experimental studies provided evidence that a reduction of Cx43 expression is critical to increase the propensity for ventricular tachyarrhythmias (17-22).

As LFN induces the development of myocardial fibrosis (8) and perivascular fibrosis (9) and taking into account that the presence of interstitial fibrosis in association with a decrease of Cx43 seems to have arrhythmogenic consequences (23-25), the importance of quantifying this protein is crucial to establish the occurrence of a morphological arrhythmogenic substrate induced by LFN. Thus, the aim of this study was to evaluate modifications on cardiac Cx43 induced by LFN in Wistar rats.

Methods

Eighteen adult Wistar rats from a Spanish breeder (Charles River Laboratories España, SA, Spain) were studied. The animals were treated in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes and with the Portuguese legislation for the same purpose. Ten rats were continuously exposed to LFN for a period of three months. The control group of 8 rats was kept in a silent environment. All the animals were kept in cages, fed standard rat food and had free access to water.

The sound signal was emitted by an analog noise generator and the noise level was the same as previously reported (26).

The hearts were fixed in 10% buffered formalin, transversely sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected for the study.

The samples were incubated with polyclonal antibody connexin-43m (GJA1) diluted 1:1000 overnight at 4º C for immunohistochemical analysis.

The histological images were acquired with an optical microscope using 400 x magnification. In each section the optical fields were selected from the left ventricle, the interventricular septum and the right ventricle. Criteria used to select each field were defined by the myocardium portions containing the highest visualization of Cx43 immunostained intercalated disks. A total of 146 optical fields were selected from all the anatomical components, by three observers, under blinded assessment, and analyzed using the *Image J software* that gives a quantification based on the image colour analysis. The signal intensity threshold value of 140 on the 0 – 255 scale was identified to distinguish Cx43 from other structures. All areas with signal intensity between 0 and 140 were considered gap junctions at the intercalated disks and the following parameters were measured: 1- Cx43, 2-muscle. Then a ratio of Cx43/muscle was calculated.

Data are presented as mean ± SD. Comparisons among groups simultaneously for the three anatomical regions were performed by One-Way MANOVA, while comparisons *in totum* were performed using a t-test for independent samples. Statistical tests were applied at the 5% level of significance.

Results

The histological observation showed immunostained Cx43 at the intercalated disks and examples of sections from LFN-exposed and control rats are shown in figure 1. In general, less immunoreactive particles were observed among the samples of LFN exposed rats.

The ratio Cx43/muscle in each anatomical region is shown in table 1 and is graphically depicted in figure 2. The ratio Cx43/muscle *in totum* is also shown in table 1.

The mean values of cardiac muscle were not significantly different between the exposed and control animals in any of the anatomical regions considered in this study ($p \geq 0.664$).

The total amount of Cx43 was significantly reduced in the left ventricle ($p=0.011$) and in the right ventricle ($p=0.009$) of LFN-exposed animals when compared to controls. No differences were detected between the two groups concerning the total amount of Cx43 in the interventricular septum ($p=0.237$).

The ratio Cx43/muscle *in totum* decreased 43.3% among the LFN-exposed rats ($p=0.009$). Multivariate comparisons over the three anatomical regions showed significant

differences between groups (p=0.001) with a decrease of 46.2%, 22.2% and 55.6% respectively in the left ventricle (p=0.008), in the interventricular septum (p=0.301) and in the right ventricle (p=0.004).

Table 1 – Ratio Cx43/muscle in each anatomical region and *in totum* in LFN-exposed (n = 10) and control (n = 8) animals

Anatomical region	Group	Mean	Std. Deviation	Percent decrease with LFN exposure	p
LV	exposed	0.014	0.006	46.2%	0.008
	control	0.026	0.008		
IVS	exposed	0.014	0.006	22.2%	0.301
	control	0.018	0.008		
RV	exposed	0.016	0.012	55.6%	0.004
	control	0.036	0.012		
In totum	exposed	0.015	0.007	43.3%	0.009
	control	0.026	0.009		

LV = Left Ventricle; IVS = Interventricular Septum ; RV = Right Ventricle; LFN = Low- Frequency Noise

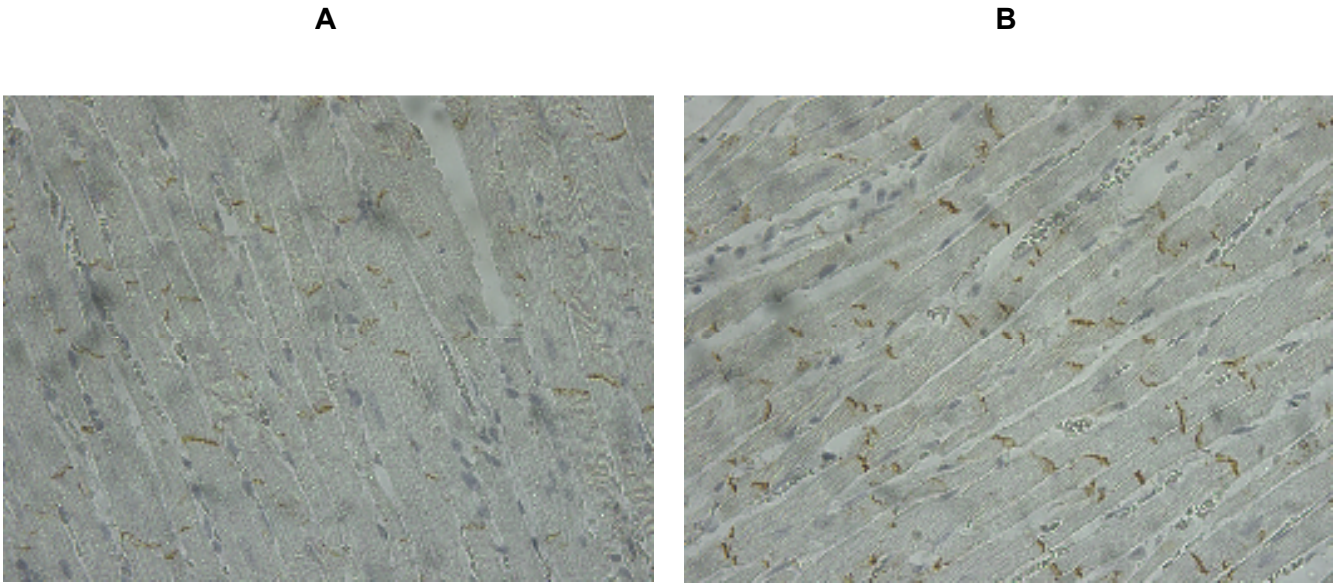


Figure 1 – Immunostained connexin43 observed at the intercalated disks in a section taken from the left ventricle of a LFN-exposed rat (A) and control rat (B) (x 400).

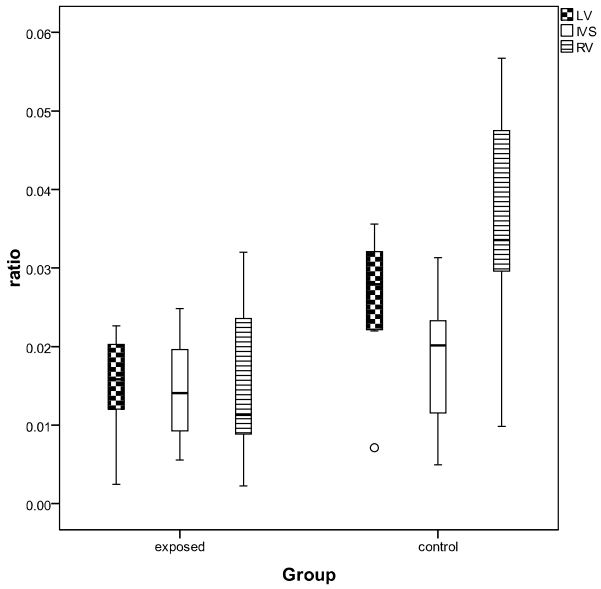


Figure 2 – Ratio Cx43/muscle in the left ventricle (LV), interventricular septum (IVS) and right ventricle (RV) in LFN exposed and control animals. A significant reduction was observed in exposed animals in the left ventricle ($p = 0.008$) and in the right ventricle ($p = 0.004$) but not in the interventricular septum ($p = 0.301$).

Discussion

As far as we know this is the first study concerning the evaluation of cardiac Cx43 in rats submitted to LFN.

In humans, gap junction remodeling has been studied in several pathologies and arrhythmias (10-16, 27, 28). The loss of Cx43 expression has been shown to be a key for the development of an arrhythmic anatomic substrate in chronically hypertrophied myocardium (15). Modifications in gap junction organization and on Cx43 expression contribute to conduction disturbances and development of arrhythmias in myocardial infarction (27), non-ischemic dilated cardiomyopathy (12, 13), cardiac heart failure (11) and valvular heart disease (14).

In several studies, a reduction of Cx43 from 30 to 50% occurs in ventricular remodeling (14, 23, 29), but changes in gap junction expression alone are presumably not sufficient for conduction slowing and enhanced arrhythmogenicity, apparently because there is a large conduction reserve (30).

Meanwhile, it is known that an increase of the intercellular collagen deposits may lead to anisotropic reentry (23, 24), and a strong enhancement of arrhythmogenic vulnerability can be attained by the association of increased fibrosis with a 50% reduction of Cx43 expression (31).

As LFN induces the development of interstitial myocardial fibrosis (8) and perivascular fibrosis (9) we hypothesized that the finding of significant modifications on gap junctions after LFN exposure could lead to a morphological arrhythmogenic substrate.

In our study the measurement of Cx43 was performed in equivalent tissue mass among exposed and control animals and showed a significant reduction in rats exposed to LFN. The modification was evident at the free ventricular wall but not in the interventricular septum suggesting that this anatomical region could be more protected against the effects of LFN. However, this does not discard the possibility of a strong alteration of the electrophysiological *milieu*. In fact, the observed statistical powers of 99% for the multiple comparisons between groups and in excess of 80% for the comparisons between groups regarding the left and right ventricles are noteworthy, in view of the sample dimension, suggesting the marked effects of LFN exposure.

Taking into consideration the universal existence of LFN in modern societies and having in mind the difficulties to explain some ventricular tachyarrhythmias without structural heart disease, the hypothesis of idiopathic ventricular fibrillation as being also a

consequence of gap junction remodeling mediated through the effects of LFN, should not be despicable. On the other hand, in patients with already known specific cardiac disorders, a reduction of gap junctions by the exposure to LFN makes the development of arrhythmic events possible, carrying out an adverse prognosis.

We still do not know the mechanisms underlying the fibrotic development we previously reported in rats exposed to LFN (8) or to industrial noise (9) as well as the mechanism of the Cx43 alteration observed in this study. Nevertheless, the Cx43 remodeling has been linked to intrinsic factors occurring on biosynthesis at transcriptional or postranscriptional phase (32-33). As connexin43 is degraded through lysosomal or proteasomal pathway (34-35) one can also speculate that the Cx43 reduction observed in this study can be related to an activation of these pathways. Theoretically, LFN acting as an external mechanical force could also lead to activation of protein kinases which might modify the level of Cx43 phosphorylation (36, 37).

Regardless of the mechanism how LFN induces loss of Cx43, our results suggest that the significant reduction of this protein can lead to electrophysiological modifications. The occurrence of a significant myocardial and periarterial fibrosis induced respectively by LFN and industrial noise reported earlier by our group (8, 9), together with a possible gap junction remodeling observed in this study, makes the development of a morphological arrhythmogenic substrate by LFN possible. Further experimental and clinical studies are needed to evaluate the functional and arrhythmic consequences. With this study we put forward the hypothesis of a link between LFN and ventricular tachyarrhythmias, opening a new investigational area concerning the effects of LFN on the heart.

In conclusion, we can state that low frequency noise induces modifications on cardiac connexin43 in rats. The connexin43 reduction observed in our study can contribute to a morphological arrhythmogenic substrate.

Footnotes

Conflict of interest: none declared

References

1- Alves-Pereira M, Castelo Branco NAA. Vibroacoustic disease: biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signalling. Prog Bioph Mol Biol 2007; 93: 256 – 279.

2- da Fonseca J, dos Santos JM, Branco NC, Alves-Pereira M, Grande NR, Oliveira P, Martins AP. Noise-induced gastric lesions: a light and scanning electron microscopy study of the alterations of the rat gastric mucosa induced by low frequency noise. Cent Eur J Public Health 2006; 14: 35 – 38.

3- Grande NR, Águas AP, Sousa Pereira A, Monteiro E, Castelo Branco NAA. Morphological changes in the rat lung parenchyma exposed to low frequency noise. Aviat Space Environ Med 1999; 70: A70 – A77.

4- de Sousa Pereira A, Águas AP, Grande NR, Mirones J, Monteiro E, Castelo Branco NAA.The effect of chronic exposure to low frequency noise on rat tracheal epithelia. Aviat Space Environ Med. 1999; 70: A186 – A190.

5- Martins dos Santos J, Grande NR, Castelo Branco NAA, Zagalo C, Oliveira P, Alves-Pereira M. Lymphatic lesions and vibroacoustic disease. Eur J Lymphol 2004; 12: 17 – 20.

6- Oliveira PM, Pereira da Mata AD, Martins dos Santos JA, da Silva Marques DN, Branco NC, Silveira JM, Correia da Fonseca JC. Low-frequency noise effects on the parotid gland of the Wistar rat. Oral Dis 2007; 13: 468 – 473.

7- Martins dos Santos J, Grande NR, Castelo Branco NA, Zagalo C, Oliveira P. Vascular lesions and vibroacoustic disease. Eur J Anat 2002; 6: 17 – 21.

8- Antunes E, Oliveira P, Borrecho G, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Myocardial fibrosis in rats exposed to low frequency noise. Acta Cardiol 2013; 68: 241 – 245.

9- Antunes E, Oliveira P, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Histomorphometric evaluation of the coronary artery vessels in rats submitted to industrial noise. Acta Cardiol 2013; 68: 285 – 289.

10- Smith JH, Green CR, Peters NS, Rothery S, Severs NJ. Altered patterns of gap junction distribution in ischemic heart disease. An immunohistochemical study of human myocardium using laser scanning confocal microscopy. Am J Pathol 1991; 139: 871 – 878.

11- Kostin S, Rieger M, Dammer S, Hein S, Richter M, Klovekorn WP, Bauer EP, Schaper J. Gap junction remodeling and altered connexin43 expression in the failing human heart. Mol Cell Biochem 2003; 242: 135 – 144.

12- Kitamura H, Ohnishi Y, Yoshida A, Okajima K, Azumi H, Ishida A, Galeano EJ, Kubo S, Hayashi Y, Itoh H, Yokoyama M. Heterogeneous loss of connexin43 protein in nonischemic dilated cardiomyopathy with ventricular tachycardia. J Cardiovasc Electrophysiol 2002; 13: 865 – 887.

13- Kitamura H, Ohnishi Y, Yoshida A, Okajima K, Azumi H, Ishida A, Galeano EJ, Kubo S, Fukuzawa K, Takano T, Yokoyama M. Correlation of connexin43 expression and late ventricular potentials in nonischemic dilated cardiomyopathy. Circ J 2003; 67: 1017 – 1021.

14- Kostin S, Dammer S, Hein S, Klovekorn WP, Bauer EP, Schaper J. Connexin 43 expression and distribution in compensated and decompensated cardiac hypertrophy in patients with aortic stenosis. Cardiovasc Res 2004; 62: 426 – 436.

15- Peters NS, Green CR, Poole-Wilson PA, Severs NJ. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. Circulation 1993; 88: 864 – 875.

16- Takeuchi S, Akita T, Takagishi Y, Watanabe E, Sasano C, Honjo H, Kodama I. Disorganization of gap junction distribution in dilated atria of patients with chronic atrial fibrillation. Circ J 2006; 70: 575 – 582.

17- Gutstein DE, Morley GE, Tammaddon H, Vaidya D, Schneider MD, Chen J, Chien KR, Stuhlmann H, Fishman GI. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circ Res 2001; 88: 333 – 339.

18- Danik SB, Liu F, Zhang J, Suk HJ, Morley GE, Fishman GI, Gutstein DE. Modulation of cardiac gap junction expression and arrhythmic susceptibility. Circ Res 2004; 95: 1035 – 1041.

19- van Rijen HVM, Eckardt D, Degen J, Theis M, Ott T, Willecke K, Jonsma HJ, Opthof T, Bakker JMT. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. Circulation 2004; 109: 1048 – 1055.

20- Uzzaman M, Honjo H, Tagagishi Y, Emdad L, Magee AI, Severs NJ, Kodama I. Remodeling of gap junctional coupling in hypertrophied right ventricles of rats with monocrotaline-induced pulmonary hypertension. Circ Res 2000; 86: 871 – 879.

21- Mayama T, Matsumura K, Lin H, Ogawa K, Imanaga I. Remodeling of cardiac gap junction connexin 43 and arrhythmogenesis. Exp Clin Cardiol 2007; 12: 67 – 76.

22- Lin H, Ogawa K, Imanaga I, Tribulova N. Remodeling of connexin 43 in the diabetic rat heart. Mol Cell Biochem 2006; 290: 69 – 78.

23- Peters NS, Coromilas J, Severs NJ, Wit AL. Disturbed connexin43 gap junction distribution correlates with the location of reentrant circuits in the epicardial border zone of healing canine infarcts that cause ventricular tachycardia. Circulation 1997; 97: 988 – 996.

24- Kawara T, Derksen R, de Groot JR, Coronel R, Tasseron S, Linnenbank AC, Hauer RN, Kirkels H, Janse MJ, de Bakker JM. Activation delay after premature stimulation in chronically diseased human myocardium relates to the architecture of interstitial fibrosis. Circulation 2001; 104: 3069 – 3075.

25- La Vecchia L, Ometto R, Bedogni F, Finocchi G, Mosele GM, Bozzola L, Bevilacqua P, Vincenzi M. Ventricular late potentials, interstitial fibrosis, and right ventricular function in patients with ventricular tachycardia and normal left ventricular function. Am J Cardiol 1998; 81: 790 – 792.

26- da Fonseca J, Martins dos Santos J, Castelo-Branco N, Alves-Pereira M, Grande N, Oliveira P. Noise-induced duodenal lesions. Eur J Anat 2005; 9: 29 – 33.

27- Kieken F, Mutsaers N, Dolmotova E, Virgil K, Wit AL, Kellezi A, Hirst-Jensen BJ, Duffy HS, Sorgen PL. Structural and molecular mechanisms of gap junction remodeling in epicardial border zone myocytes following myocardial infartion. Circ Res 2009; 104: 1103 – 1112.

28- Basso C, Czarnowska E, Barbera ML, Bauce B, Beffagna G, Wlodarska EK, Pilichou K, Ramondo A, Lorenzon A, Wozniek O, Corrado D, Daliento L, Danieli GA, Valente M, Nava A, Thiene G, Rampazzo A. Ultrastructural evidence of intercalated disc remodeling in arrhythmogenic right ventricular cardiomyopathy: an electron microscopy investigation on endomyocardial biopsies. Eur Heart J 2006; 27: 1847 – 1854.

29- Kaprielian RR, Gunning M, Dupont E, Sheppard MN, Rothery SM, Underwood R, Pennell DJ, Fox K, Pepper J, Poole-Wilson PA, Severs NJ. Downregulation of

immunodetectable connexin43 and decreased gap junction size in the pathogenesis of chronic hibernation in the human left ventricle. Circulation 1998; 97: 651 – 660.

30- van Rijen, de Bakker JM, van Veen TA. Hypoxia, electrical uncoupling, and conduction slowing: Role of conduction reserve. Cardiovasc Res 2005; 66: 9 – 11.

31- Jansen JA, van Veen AA, Bosch AA, van der Nagel R, Vos MA, Bakker JM, van Rijen HV. Arrhythmia vulnerability of aged haploinsufficient Cx43 mice is determinant by heterogeneous downregulation of Cx43 combined with increased fibrosis. Circulation 2008; 118: S494.

32- Teunissen BE, Bierhuisen MF. Transcriptional control of myocardial connexins. Cardiovasc Res 2004; 62: 246 – 255.

33- Yang B, Lin H, Xiao J, , Lu Y, Luo X, Li B. T, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. The muscle-specific micro-RNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 2007; 13: 486 – 491.

34- Laing JG, Tadros PN, Green K, Saffitz JE, Beyer EC. Proteolysis of connexin43-containing gap junctions in normal and heat-stressed cardiac myocytes. Cardiovasc Res 1998; 38: 711 – 718.

35- Berthoud VM, Minogue PJ, Laing JG, Beyer EC. Pathways for degradation of connexins and gap junctions. Cardiovasc Res 2004; 62: 256 – 267.

36- Saffitz JE, Kleber AG. Effects of mechanical forces and mediators of hypertrophy on remodeling of gap junction in the heart. Circ Res 2004: 94: 585 – 591.

37- Yamada K, Green KG, Samarel AM, Saffitz JE. Distinct pathways regulate expression of cardiac electrical and mechanical junction proteins in response to stretch. Circ Res 2005; 97: 346 – 353.

5. EFFECTS OF LOW-FREQUENCY NOISE ON CARDIAC COLLAGEN AND CARDIOMYOCYTE ULTRASTRUCTURE: AN IMMUNOHISTOCHEMICAL AND ELECTRON MICROSCOPY STUDY

Eduardo Antunes [(1)], Gonçalo Borrecho [(1)], Pedro Oliveira [(1)], António P. Alves de Matos [(1)], José Brito [(1)], Artur Águas [(2)], José Martins dos Santos [(1)]

(1) *Center for Interdisciplinary Research Egas Moniz, Health Sciences Institute, Monte de Caparica, Portugal*

(2) *Department of Anatomy and UMIB of ICBAS, Abel Salazar Institute for Biomedical Sciences, University of Porto, Porto, Portugal*

Accepted in the International Journal of Clinical and Experimental Pathology in 2013

Abstract

Introduction: Low-frequency noise (LFN) leads to the development of tissue fibrosis. We previously reported the development of myocardial and perivascular fibrosis and a reduction of cardiac connexin43 in rats, but data is lacking concerning the affected type of collagen as well as the ultrastructural myocardial modifications. **Objectives:** The aim of this study was to quantify cardiac collagens I and III and to evaluate myocardial ultrastructural changes in Wistar rats exposed to LFN. **Methods:** Two groups of rats were considered: A LFN-exposed group with 8 rats continuously submitted to LFN during 3 months and a control group with 8 rats. The hearts were sectioned and the mid-ventricular fragment was selected. After immunohistochemical evaluation, quantification of the collagens and muscle were performed using the *image J software* in the left ventricle, interventricular septum and right ventricle and the collagen I/muscle and collagen III/muscle ratios were calculated. Transmission electron microscopy (TEM) was used to analyze mid-ventricular samples taken from each group. **Results:** The collagen I/muscle and collagen III/muscle ratios increased *in totum* respectively 80% ($p<0.001$) and 57.4% ($p<0.05$) in LFN-exposed rats. TEM showed interstitial collagen deposits and changes in mitochondria and intercalated discs of the cardiomyocytes in LFN-exposed animals. **Conclusions:** LFN increases collagen I and III in the extracellular matrix and induces ultrastructural alterations in the cardiomyocytes. These new morphological data open new and promising paths for further experimental and clinical research regarding the cardiac effects of low-frequency noise.

Key-words: Low-frequency noise; Collagen I and III; Myocardial ultrastructure; Immunohistochemistry; Transmission electron microscopy.

Introduction

Low-frequency noise (LFN) is characterized by large pressure amplitude (≥ 90 dB SPL) and low frequency bands (≤ 500 Hz) and leads to an abnormal proliferation of collagen and development of tissue fibrosis (1-6). We previously reported a significant fibrotic development in ventricular myocardium of rats exposed to LFN (7) and an increase of perivascular fibrosis in the arterial coronary vessels after exposure to industrial noise (8). Modifications on the electrophysiological *milieu,* characterized by a significant reduction of connexin43 in rats exposed to LFN were also observed in a recent study performed by our group (9). From the results of our studies we put forward that the universal existence of LFN in modern societies can contribute to aggravate preexisting cardiac diseases or even explain some idiopathic cardiomyopathies and ventricular tachyarrhythmias. Consequently, looking into new morphological data, one can establish more bridges to experimental and clinical investigations.

In the heart, type I and III collagens represent respectively 85% and 11% of the extracellular matrix collagen composition and the effects of LFN on each one are not known. Also, the ultrastructural changes induced by LFN in the cardiomyocyte are not defined.

The aim of this study was to perform an immunohistochemical quantification of collagen I and III and to evaluate the ultrastructure of the ventricular myocardium in rats exposed to low-frequency noise.

Methods

Sixteen adult Wistar rats were studied. The animals were treated in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes and with the Portuguese legislation for the same purpose. Eight rats were continuously exposed to LFN for a three-month period. The control group of 8 rats was kept in a silent environment. All the animals were kept in cages, fed standard rat food and had free access to water. The sound spectrum emanating from an analog noise generator was similar to the previously reported (10).

After the rats were sacrificed with an intraperitoneal injection of a lethal dose of sodium pentobarbital, the hearts were removed, fixed in 10% buffered formalin, transversely sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected for the study. The fragments were dehydrated with progressive graded ethanol

series, cleared with xylene and embedded in paraffin. The paraffin blocks were sliced into sections with 3.5 micrometers and mounted in glass slides and after deparaffinization and rehydration the endogenous peroxidase activity was blocked with use of 3% H_2O_2 for 10 minutes. Then, sections were incubated overnight at room temperature with polyclonal antibodies to collagen I and III diluted 1:500 for immunohistochemical analysis. The slides were finally counterstained with hematoxylin, dehydrated and mounted.

The histological images were obtained with an optical microscope using 400 x magnifications. In each section the optical fields were selected from the left ventricle, the interventricular septum and the right ventricle. Criteria used to select each field were defined by the myocardial samples containing the highest visualization of immunostained collagen I and III. A total of 132 optical fields were selected from all the anatomical components, by three observers, under blinded assessment, and analyzed using the *Image J software.* The signal intensity threshold value of 140 on the 0 – 255 scale was identified to distinguish collagen from other structures and the collagen I/muscle and collagen III/muscle ratios were calculated.

Concerning the ultrastructure evaluation and as a preliminary illustrating purpose, samples from mid-ventricular segments of five rats were cut in fragments of less than 1 mm^3 and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, followed by 1% osmium tetroxide in the same buffer and uranyl acetate 0.5% in acetate acetic acid buffer 0.1M, pH 5. After dehydration in ethanol and passage epoxypropane, the samples were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined and photographed in a transmission electron microscope.

Statistical analysis

Data are presented as mean ± SE. In order to assess the differences between LFN exposed and control animals concerning the collagen to muscle ratios in each anatomical region and *in totum*, MANOVA model was fit to the data. A p value < 0.05 was considered statistically significant.

Results

The histological observations showed immunostained collagen I and III in the extracellular matrix. Examples from LFN-exposed and control rats are shown in figures 1 and 2 respectively for collagen I and III. Histological observations showed diffuse interstitial deposits of collagen in LFN-exposed rats.

Prior to the analysis of the ratios, the assumption of homogeneous variance-covariance matrix was validated by the M-Box test (p = 0.593). This multivariate approach to the data showed that there were marked differences between exposed and non-exposed animals for the selected anatomical regions and *in totum*, as expressed by the statistical significance achieved for the collagen I/muscle ratio (p = 0.001) with an observed power of 99.6% and for the collagen III/muscle ratio (p = 0.021) with an observed power of 79.6%.

The results obtained for the collagen I/muscle ratio for each of the anatomical regions and *in totum,* are showed in table 1 and figure 3. The percent increase with LFN exposure was 80% (p<0.001) *in totum* and 76% (p=0.055), 105% (p=0.046) and 84% (p=0.032) respectively for the left ventricle, interventricular septum and right ventricle.

For the collagen III/muscle ratio the results obtained are presented in table 2 and figure 3. In this case differences were detected at the interventricular septum and *in totum.* The percent increase with LFN exposure was 57.4% (p=0.027) *in totum* and 90.9% (p=0.063), 85.7% (p=0.006) and 31.6% (p=0.206) respectively for the left ventricle, interventricular septum and right ventricle.

Examples of the ultrastructural analysis by TEM are shown in figures 4, 5 and 6. In LFN-exposed rats the ultrastructural evaluation confirmed extracellular matrix alterations, characterized by accumulation of collagen. The cardiomyocytes are pushed apart with no visible gap junctions at the interplicate regions of the intercalated discs. Additionally, a considerable number of enlarged mitochondria and some lipofuscin granules were observed.

Table 1- Collagen I/muscle ratio in each anatomical region and *in totum* in LFN-exposed (n = 8) and control (n = 8) animals

Anatomical region	Group	Mean	Std. error	Percent increase with LFN exposure	P
LV	Exposed Control	0.044 0.025	0.006 0.006	76%	0.055
IVS	Exposed Control	0.037 0.018	0.006 0.006	105%	0.046
RV	Exposed Control	0.059 0.032	0.008 0.008	84%	0.032
in totum	Exposed Control	0.045 0.025	0.002 0.002	80%	< 0.001

LV = Left Ventricle; IVS = Interventricular Septum; RV = Right Ventricle; LFN = Low-Frequency Noise

Table 2 – Collagen III/muscle ratio in each anatomical region and *in totum* in LFN-exposed (n = 8) and control (n = 8) animals

Anatomical region	Group	Mean	Std. error	Percent increase with LFN exposure	P
LV	Exposed	0.084	0.014	90.9%	0.063
	Control	0.044	0.014		
IVS	Exposed	0.091	0.009	85.7%	0.006
	Control	0.049	0.009		
RV	Exposed	0.154	0.020	31.6%	0.206
	Control	0.117	0.020		
in totum	Exposed	0.107	0.011	57.4%	0.027
	Control	0.068	0.011		

LV = Left Ventricle; IVS = Interventricular Septum; RV = Right Ventricle; LFN = Low-Frequency Noise

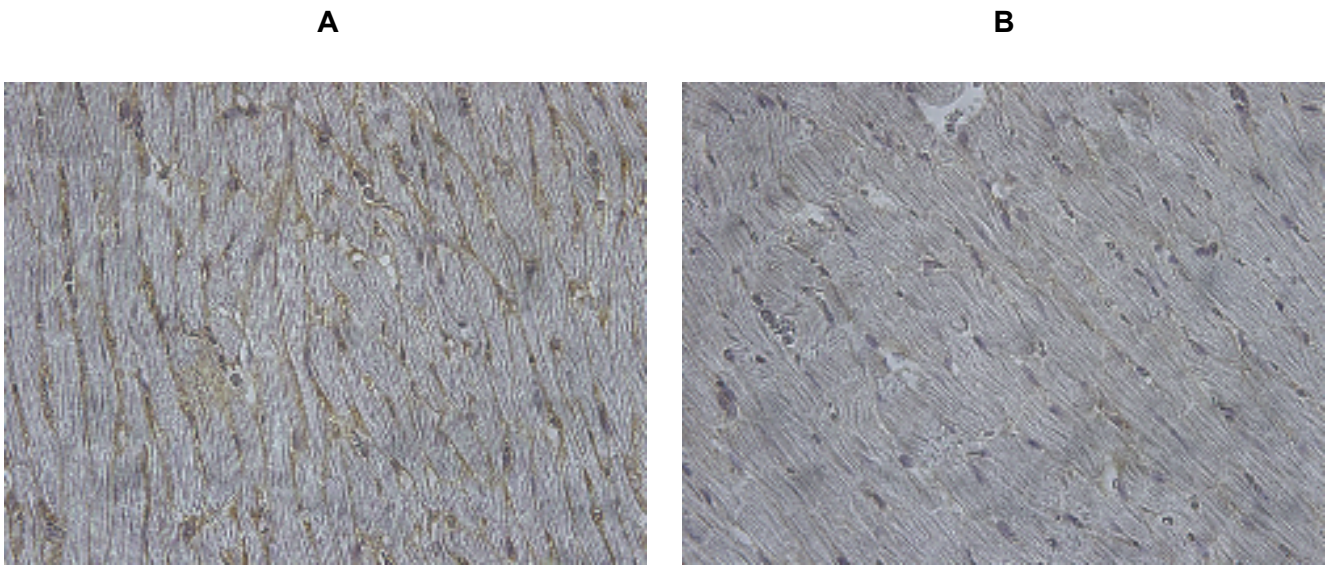


Figure 1 – Immunostained collagen I in a section taken from the left ventricle of a LFN-exposed rat (A) and control rat (B). Immunoreactive type I collagen appears brown between the cardiomyocytes (x 400).

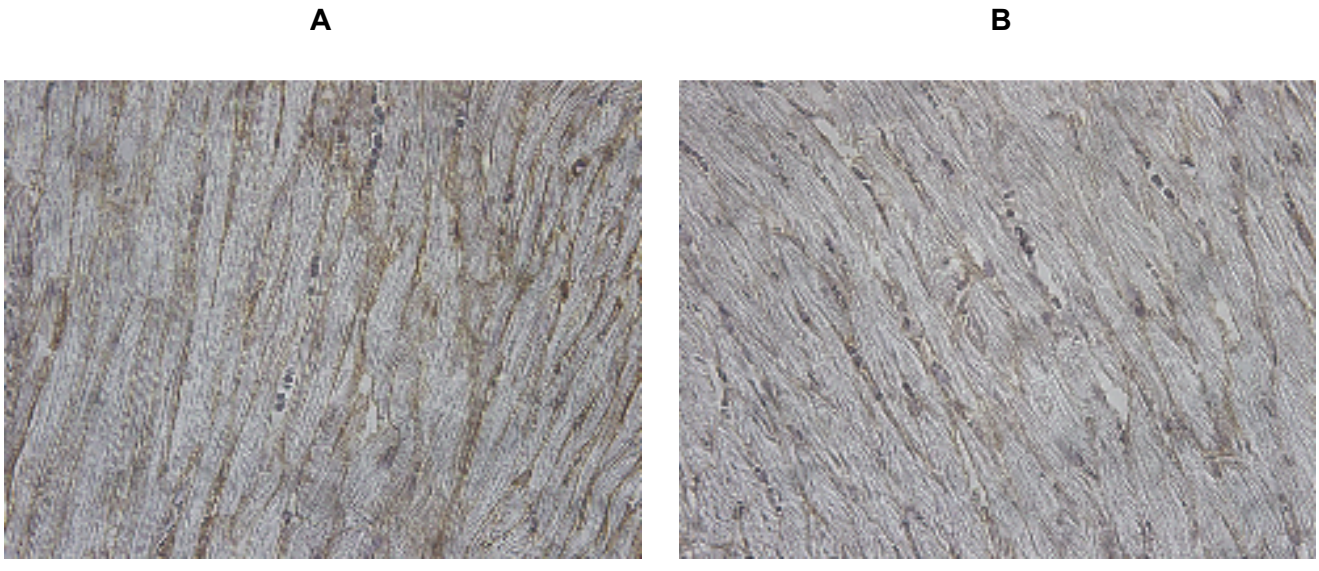
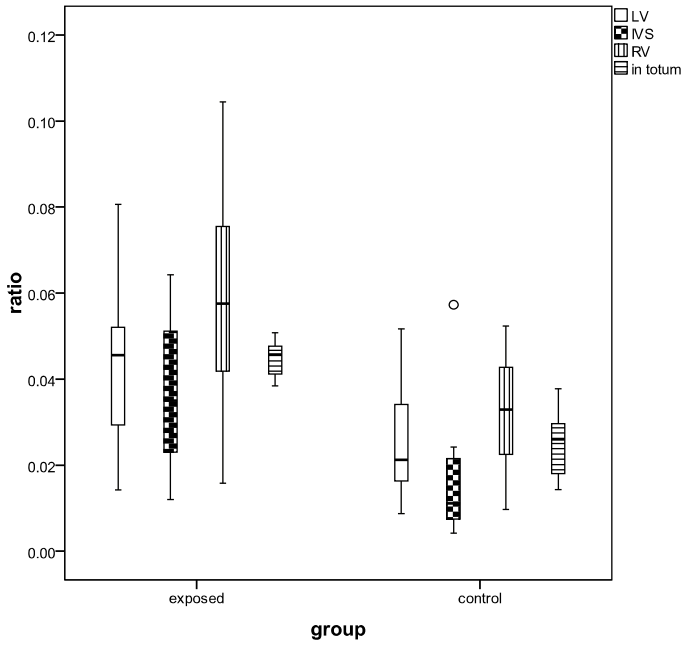


Figure 2 – Immunostained collagen III in a section taken from the left ventricle of a LFN-exposed rat (A) and control rat (B). Immunoreactive type III collagen appears brown between the cardiomyocytes (x 400).

A



B

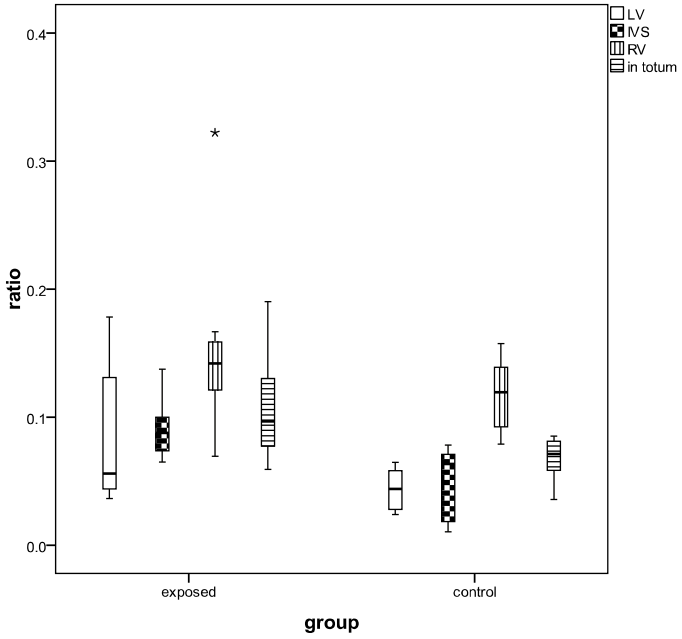


Figure 3 – Collagen I/muscle ratio (A) and collagen III/muscle ratio (B) in the left ventricle (LV), interventricular septum (IVS), right ventricle (RV) and *in totum* in LFN exposed and control animals.

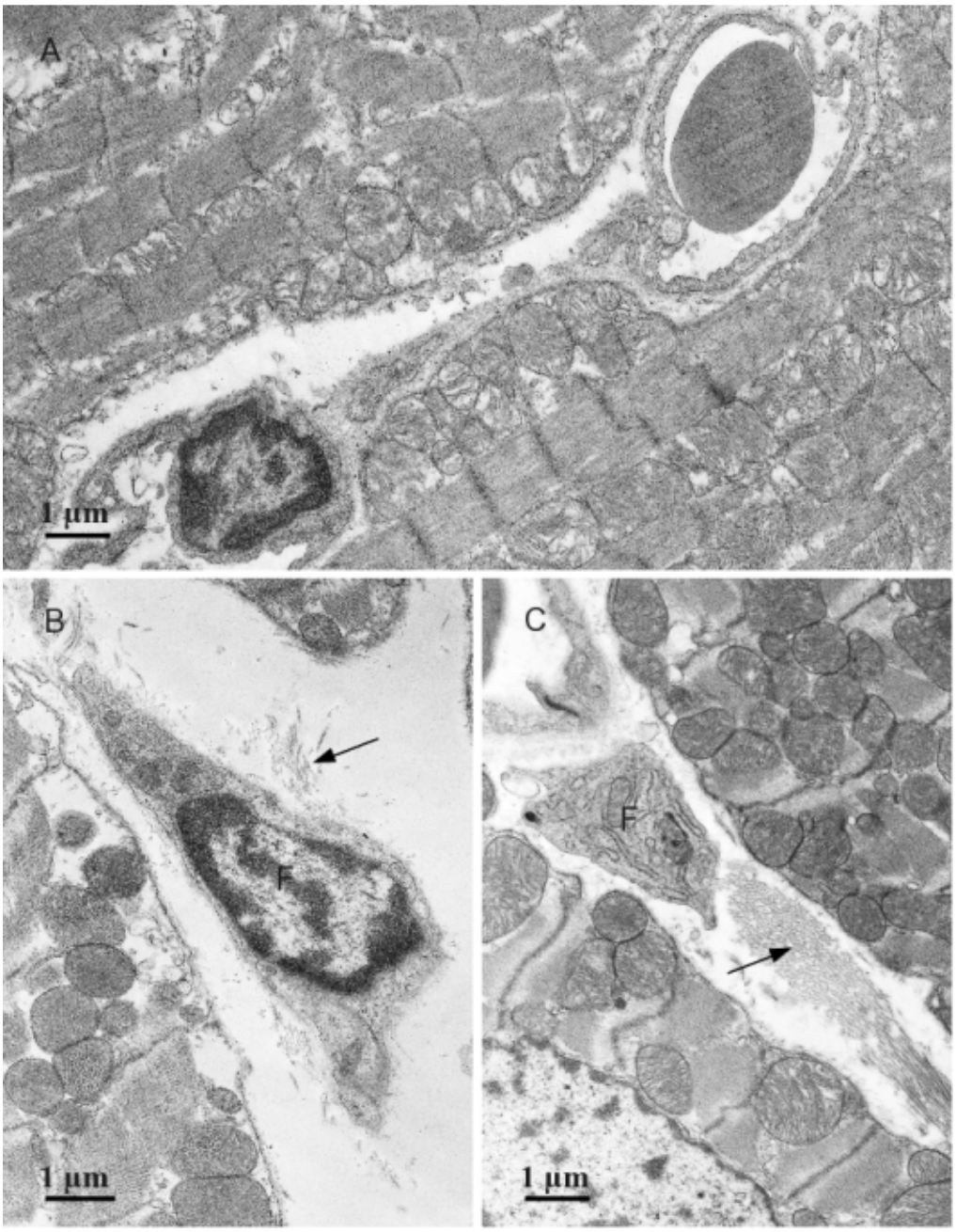


Figure 4 - Electron micrograph (TEM) of a section of ventricular muscle. (A) Control rat; (B) and (C) LFN-exposed rats. Fibroblasts (F) surrounded by collagen (arrows) are observed in the interstitium of the LFN-exposed rats.

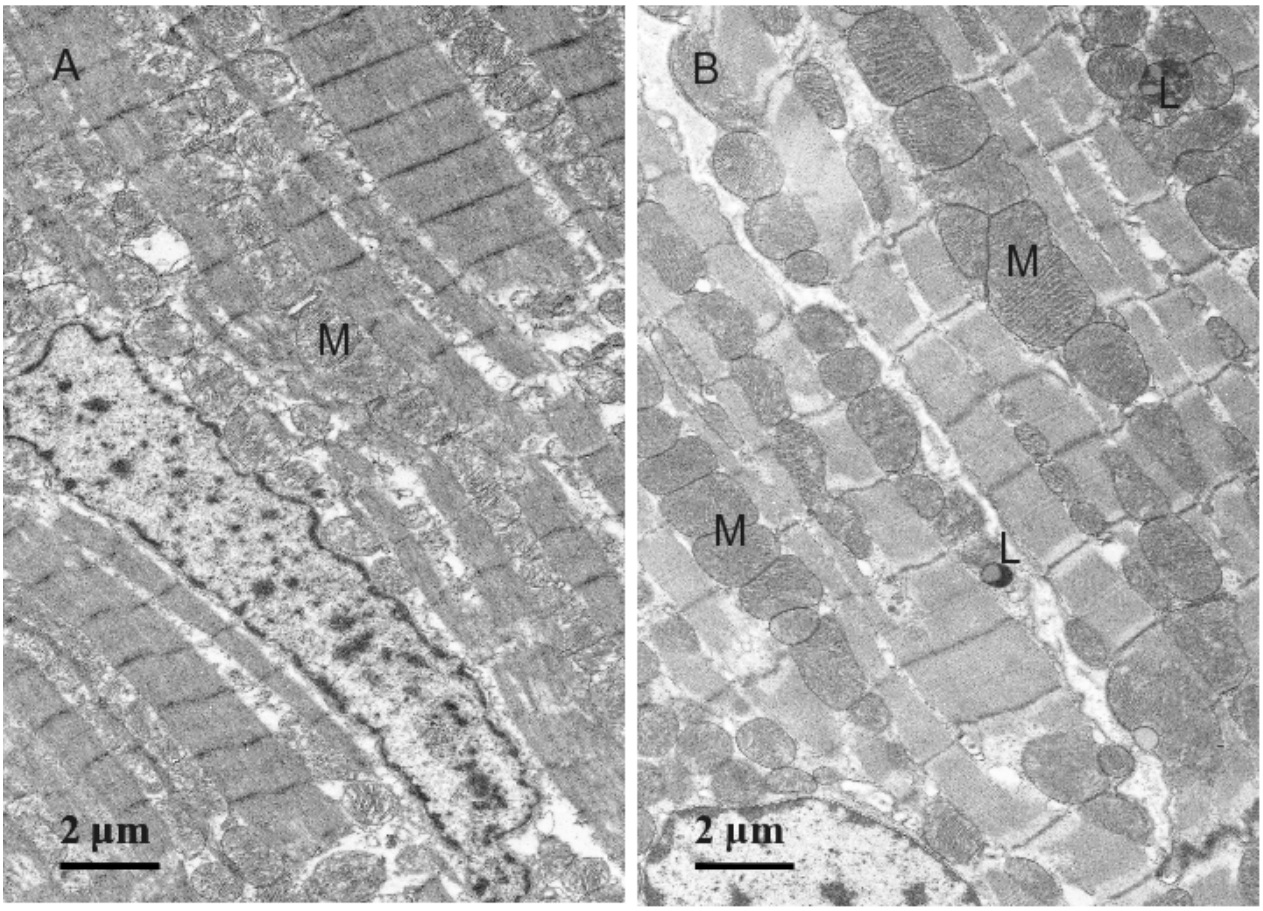


Figure 5 - Electron micrograph (TEM) of a longitudinal section of ventricular muscle. (A) Control rat; (B) LFN-exposed rat. Numerous enlarged mitochondria surrounding the sarcomeres are observed in the LFN-exposed rat (M). Lipofuscin granules are also observed in LFN-exposed rats (L).

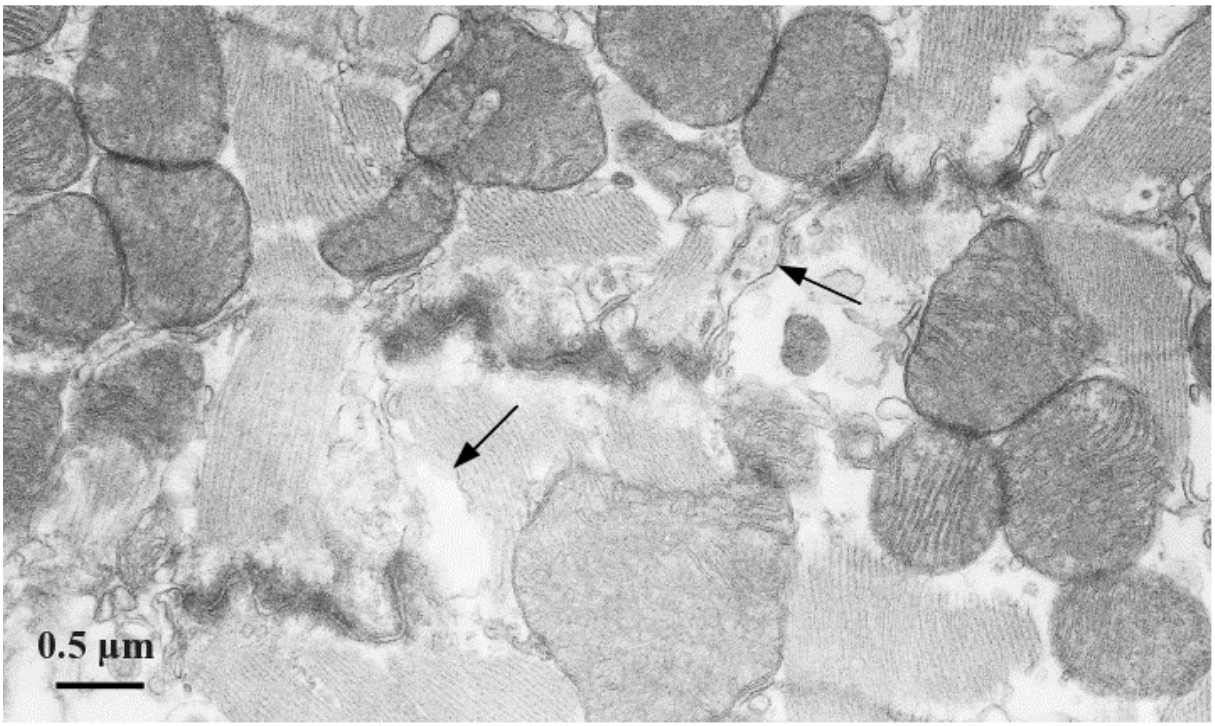


Figure 6 - Electron micrograph (TEM) section of a LFN-exposed ventricular myocardium, showing a typical steplike intercalated disc and numerous mitochondria surrounding the sarcomeres. Cell membrane separation (arrows) is observed in the interplicate region of the intercalated disc.

Discussion

Our study analyzes the cardiac type I and III collagens and the ultrastructure of the ventricular myocardium in rats exposed to LFN. Type I collagen is responsible for the tensile strength, while type III contributes to the elasticity of the myocardium. A correct balance between the synthesis and degradation of these collagens is necessary to maintain myocardial structure and function. Fibrosis represents an abnormal deposition of collagen and can lead to myocardial stiffness and diastolic dysfunction (11, 12) and in addition, modifies the electrophysiological myocardium properties facilitating the occurrence of ventricular arrhythmias (13, 14).

In our study, a significant increase of collagen I and III was observed. An increase was shown in all the studied anatomical areas but some did not reach statistical significance.

The ultrastructural observation denoted high concentration of collagen in the extracellular matrix next to fibroblasts, confirming the pronounced effect of LFN on the connective tissue. Another relevant modification was observed in the cardiomyocytes by the presence

of numerous enlarged mitochondria. In a previous study performed in rats submitted to infrasound (15), swelled mitochondria were observed, explained by a possible cellular membranous direct damage, leading to an overload of Ca^{2+} and consequent inhibition of the oxidative phosphorylation, followed by a reduction of the cardiomyocyte energy. In contrast, in our study, an increase of the mitochondria number and size was observed, being the main organelle alteration in response to LFN. This new data may suggest high-energy activity in cardiomyocytes and suggests that cardiac morphological changes induced by LFN may not be confined to the extracellular matrix.

Corroborating the hypothesis of a direct deleterious LFN action on the cardiomyocyte are the results we previously reported showing a reduction of cardiac connexin43 after LFN exposure (9). However, the TEM observations in the present study, of cardiomyocyte separated cell membranes, puts forward the hypothesis of gap junction disruption, possibly caused by an interstitial fibrotic development. Further studies are needed to confirm these data.

Meanwhile, the immunohistochemical demonstration of an augmented collagen as well as the ultrastructural evidence of relevant collagen deposits, follow the line of our previous morphological studies showing a significant myocardial fibrotic development induced by LFN (7). We believe that this acquired experimental knowledge on extracellular matrix modification should constitute the support for clinical research applied to people exposed to LFN.

In fact, myocardial fibrosis can lead to clinical consequences such as reduced coronary flow reserve, ventricular diastolic dysfunction and ventricular tachyarrhythmias. Coronary perivascular fibrosis can limit vessel distensibility and impair coronary blood flow (16, 17). Interstitial myocardial fibrosis has deleterious effects on diastolic function through an increased stiffness and reduction of elasticity of the ventricular myocardium (11, 12, 18). Additionally, severe ventricular tachyarrhythmias may develop through reentrant phenomena induced by the electrophysiological heterogeneities in consequence of myocardial fibrosis (19 – 21).

Considering our previous and these present results, we can stress the importance of the clinical diagnosis and characterization of myocardial fibrosis as well as the study of its possible arrhythmic consequences. These issues deserve further clinical research in populations exposed to LFN.

One possible way could be the use of biochemical markers for myocardial fibrosis. In fact, high serum levels of procollagen type III aminoterminal peptide were shown in

hypertensive patients (12), in dilated cardiomyopathy (22) in hypertrophic cardiomyopathy (23) in congenital heart disease (24) and in heart failure (25, 26). Excessive levels of procollagen type I carboxyterminal peptide was found in hypertensive patients (12, 27). The relation however between these biomarkers and the myocardial fibrosis induced by LFN is not known. Nevertheless, and taking into account the significant increase of collagen I and III found in our study, we do not discard a potential clinical application of applying specific fibrosis biomarkers to evaluate populations exposed to low-frequency noise.

Bearing in mind a possible link between modifications of biochemical markers for myocardial fibrosis and ventricular arrhythmogenesis (28) and hypothesizing that LFN can induce a morphological arrhythmogenic substrate (9) the use of specific biomarkers to identify LFN-exposed individuals or patients more prone to develop ventricular arrhythmias should be considered.

Another relevant clinical implication can be the use of echocardiography to evaluate the ventricular diastolic function in people exposed to LFN. In this regard a study performed in aerospace maintenance workers showed significant alterations of the E/A ratio reflecting changes in the ventricular diastolic function. The use of the echocardiographic parameter E/A ratio was suggested to evaluate the ventricular diastolic function and to check the health condition of people exposed to LFN (29).

In addition, cardiac magnetic resonance can also possibly be used to quantify cardiac fibrosis (30, 31). Nevertheless, its application for diffuse interstitial myocardial fibrosis showed some limitations (32).

In conclusion, LFN induces cardiac morphological changes in the extracellular matrix and in the cardiomyocyte ultrastructure. The significant increase of collagen I and III and the alteration detected at the cardiomyocyte intercalated disc together with the reduction of connexin43 reported in one of our previous studies, reinforce the hypothesis of an inducible morphological arrhythmogenic substrate. The new morphological data observed in this study open new and promising paths for experimental and clinical research regarding the cardiac effects of low-frequency noise.

Footnotes

Conflict of interest: none declared.

References

1- Oliveira P, Brito J, Mendes J, da Fonseca J, Águas A, Martins dos Santos J. Effects of large pressure amplitude low frequency noise in the parotid gland perivascular-ductal connective tissue. Acta Med Port 2013; 26: 237 – 242.

2- de Sousa Pereira A, Águas AP, Grande NR, Mirones J, Monteiro E, Castelo Branco NAA.The effect of chronic exposure to low frequency noise on rat tracheal epithelia. Aviat Space Environ Med 1999; 70: A186 – A190.

3- Grande NR, Águas AP, Sousa Pereira A, Monteiro E, Castelo Branco NAA. Morphological changes in the rat lung parenchyma exposed to low frequency noise. Aviat Space Environ Med 1999; 70: A70 – A77.

4- Martins dos Santos J, Grande NR, Castelo Branco NAA, Zagalo C, Oliveira P, Alves-Pereira M. Lymphatic lesions and vibroacoustic disease. Eur J Lymphol 2004; 12: 17 – 20.

5- Fonseca J, Martins dos Santos J, Oliveira P, Laranjeira N, Aguas A, Castelo-Branco N. Noise-induced gastric lesions: a light and electron microscopy study of the rat gastric wall exposed to low frequency noise. Arq Gastroenterol 2012; 49: 82 – 88.

6- Martins dos Santos J, Grande NR, Castelo Branco NA, Zagalo C, Oliveira P. Vascular lesions and vibroacoustic disease. Eur J Anat 2002; 6: 17 – 21.

7- Antunes E, Oliveira P, Borrecho G, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Myocardial Fibrosis in Rats Exposed to Low Frequency Noise. Acta Cardiol 2013; 68: 241 – 245.

8- Antunes E, Oliveira P, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Histomorphometric evaluation of the coronary arterial vessels in rats submitted to industrial noise. Acta Cardiol 2013; 68: 285 – 289.

9- Antunes E, Borrecho G, Oliveira P, José Brito J, Águas A, Martins dos Santos J. Immunohistochemical evaluation of cardiac connexin43 in rats exposed to low-frequency noise. Int J Clin Exp Pathol 2013; 6: 1874 – 1879.

10- Oliveira PM, Pereira da Mata AD, Martins dos Santos JA, da Silva Marques DN, Branco NC, Silveira JM, Correia da Fonseca JC. Low-frequency noise effects on the parotid gland of the Wistar rat. Oral Dis 2007; 13: 468 – 473.

11- Janicki JS, Brower GL. The role of myocardial fibrillar collagen in ventricular remodeling and function. J Card Fail 2002; 8 (suppl): S319 – S325.

12- Martos R, Baugh J, Ledwidge M, O'Loughlin C, Conlon C, Patle A, Donnelly SC, McDonald K,. Diastolic heart Failure: evidence of increased myocardial turnover linked to diastolic dysfunction. Circulation 2007; 115: 888 – 895.

13- Spach MS, Boineau JP. Microfibrosis produces electrical load variations due to loss of side-to-side cell connections: a major mechanism of structural heart disease arrhythmias. Pacing Clin Electrophysiol 1997; 20: 397 – 413.

14- De Baker JM, von Capelle FJ, Janse MJ, Tasseron S, Vermeulen JT, de Jonge N, Lahpor JR. Slow conduction in the infarted human heart. 'Zigzag 'course of activation. Circulation 1993; 88: 915 – 926.

15- Pei Z, Sang H, Li R, Xiao P, He J, Zhuang Z, Zhu M, Chen J, Ma H. Infrasound-induced hemodynamics, ultrastructure, and molecular changes in the rat myocardium. Environ Toxicol 2007; 22: 169 – 175.

16- Isoyama S, Ito N, Satoh K, Takishima T. Collagen deposition and the reversal of coronary reserve in cardiac hypertrophy. Hypertension 1992; 20: 491 – 500.

17- Dai Z, Aoki T, Fukumoto Y, Shimokaxa H. Coronary perivascular fibrosis is associated with impairment of coronary blood flow in patients with non-ischemic heart failure. J Cardiol 2012; 60: 416 – 421.

18- Weber KT, Brilla CG, Janicki JS. Myocardial fibrosis. Functional significance and regulatory factors. Cardiovasc Res 1993; 27: 13 – 31.

19- Peters NS, Coromilas J, Severs NJ, Wit AL. Disturbed connexin43 gap junction distribution correlates with the location of reentrant circuits in the epicardial border zone of healing canine infarcts that cause ventricular tachycardia. Circulation 1997; 97: 988 – 996.

20- Kawara T, Derksen R, de Groot JR, Coronel R, Tasseron S, Linnenbank AC, Hauer RN, Kirkels H, Janse MJ, de Bakker JM. Activation delay after premature stimulation in chronically diseased human myocardium relates to the architecture of interstitial fibrosis. Circulation 2001; 104: 3069 – 3075.

21- La Vecchia L, Ometto R, Bedogni F, Finocchi G, Mosele GM, Bozzola L, Bevilacqua P, Vincenzi M. Ventricular late potentials, interstitial fibrosis, and right ventricular function

in patients with ventricular tachycardia and normal left ventricular function. Am J Cardiol 1998; 81: 790 – 792.

22- Klappacher G, Franzen P, Haab D, Mehrabi M, Binder M, Plesch K, Pacher R, Grimm M, Pribill I, Eichler HG. Measuring extracellular matrix turnover in the serum of patients with idiopathic or ischemic dilated cardiomyopathy and impact on diagnosis and prognosis. Am J Cardiol 1995; 75: 913 – 918.

23- Lombardi R, Betocchi S, Losi MA, Tocchetti CG, Aversa M, Miranda M, D'Alessandro G, Cacace A, Ciampi Q, Chiariello M. Myocardial collagen turnover in hypertrophic cardiomyopathy. Circulation 2003; 108: 1455 – 1460.

24- Sugimoto M, Masutani S, Seki M, Kajino H, Fujieda K, Senzaki H.High serum levels of procollagen type III N-terminal amino peptide in patients with congenital heart disease. Heart 2009; 95: 2023 – 2028.

25- Barasch E, Gottdiener JS, Aurigemma G, Kitzman DW, Han J, Kop WJ, Tracy RP. Association between elevated fibrosis markers and heart failure in the elderly. The cardiovascular health study. Circ Heart Fail 2009; 2: 303 – 310.

26- Cicoira M, Rossi A, Bonapace S, Zanolla L, Golia G, Franceschini L, Caruso B, Marino PN, Zardini P. Independent and additional prognostic value of aminoterminal propeptide of type III procollagen circulating levels in patients with chronic heart failure. J Card Fail 2004;10: 403 – 411.

27- Querejeta R, López B, González A, Sánchez E, Larman M, Martínez Ubago JL, Díez J. Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis. Circulation 2004; 110: 1263 – 1268.

28- Flevari P, Theodorakis G, Leftheriotis D, Kroupis C, Kolokathis F, Dima K, Anastasiou-Nana M, Kremastinos D. Serum markers of deranged myocardial collagen turnover: their relation to malignant ventricular arrhythmias in cardioverter-defibrillator recipients with heart failure. Am Heart J 2012; 164: 530 – 537.

29- Chao P-C, Yeh C-Y, Juang Y-J, Hu C-Y, Chen C-J. Effect of low-frequency noise on the echocardiographic parameter E/A ratio. Noise Health 2012; 14: 155 – 158.

30- Moon JC, McKenna WJ, McCrohon JA, Elliott PM, Smith GC, Pennell DJ. Toward clinical risk assessment in hypertrophic cardiomyopathy with gadolinium cardiovascular magnetic resonance. J Am Coll Cardiol. 2003; 41: 1561 – 1567.

31- Tandri H, Saranathan M, Rodriguez ER, Martinez C, Bomma C, Nasir K, Rosen B, Lima JA, Calkins H, Bluemke DA. Noninvasive detection of myocardial fibrosis in arrhythmogenic right ventricular cardiomyopathy using delayed-enhancement magnetic resonance imaging. J Am Coll Cardiol 2005; 45: 98 – 103.

32- Schalla S, Bekkers SC, Dennert R, van Suylen RJ, Waltenberger J, Leiner T, Wildberger J, Crijns HJ, Heymans S. Replacement and reactive myocardial fibrosis in idiopathic dilated cardiomyopathy: comparison of magnetic resonance imaging with right ventricular biopsy. Eur J Heart Fail. 2010;12: 227 – 231.

6. FINAL DISCUSSION AND CONCLUSIONS

This thesis focused on the evaluation of cardiac morphological changes induced by industrial and low-frequency noise (LFN) in Wistar rats. We sequentially undertook studies to analyze the coronary artery vessels (1), myocardium (2) and gap junctions (3). The fourth study evaluated collagen I and III and the cardiomyocyte ultrastructure (4).

The histomorphometric evaluation of the coronary arteries showed significant differences concerning the vessel wall-to-perivascular tissue ratio demonstrating an exuberant fibrotic perivascular tissue in the animals exposed to industrial noise (1). These changes were observed in the absence of any alteration of the internal elastic lamina or any vessel obstructive lesion, suggesting a lower susceptibility of the coronary arteries to industrial noise damage. In contrast, the perivascular fibrosis suggests that the connective tissue modification is the main consequence of industrial noise and opens the possibility of ischemia related to limitations on vessel distensibility (5).

In this regard, a study performed in patients with chronic heart failure related to non-ischemic causes, showed that coronary perivascular fibrosis may occur independently of interstitial myocardial fibrosis, leading to impairment of coronary blood flow (6). Our results showed a significant increase of perivascular fibrosis in the absence of coronary artery disease which makes it possible to admit the occurrence of ischemia through the same mechanism.

In addition, the estimated marginal means of the vessel wall-to-perivascular tissue ratio, calculated in this study (1) showed that the effects of exposure time seemed to be independent of exposure to industrial noise. Although a reduction was observed at 7 months, it remained increased among the industrial noise exposed animals. A practical illation from this result can be the need to define long-term therapeutic targets against the perivascular fibrotic development.

Concerning the evaluation of myocardial fibrosis (2) a marked development of interstitial fibrosis was observed in both ventricles and interventricular septum without important differences between anatomical regions suggesting a general myocardial fibroblastic response induced by LFN. Again this result agrees with the statement that abnormal proliferation of collagen is the main consequence induced by LFN in biological tissues.

From a theoretical point of view we hypothesized that the mechanism underlying myocardial fibrosis could be related to a loss of regulation between profibrotic and

antifibrotic molecules due to mechanical and/or neuro-humoral factors as occurs in hypertensive heart disease (7).

Whatever the fibrotic proliferation mechanisms could be, myocardial ventricular fibrosis reflects an excess of collagen in the extracellular matrix and can have important clinical consequences (7 - 10) related to ventricular dysfunction, changes in coronary flow reserve and ventricular arrhythmias (5, 6, 11 - 13).

Data is lacking concerning the development of ventricular arrhythmias in people exposed to LFN. Nevertheless, fibrosis induces stiffness of the myocardial tissue (11) and may lead to electrophysiological heterogeneities that may facilitate the appearance of ventricular arrhythmias (14). Thus, individuals exposed to LFN may, in theory, develop ventricular tachyarrhythmias.

In contrast, some data already exists concerning the evaluation of ventricular function. As described in chapter 5, in a study performed in aeronautic maintenance workers the echocardiographic parameter E/A ratio was used and significant alterations were found in individuals exposed to LFN (15). Thus, the echocardiographic evaluation can be an important tool to be undertaken in LFN-exposed professions.

The immunohistochemical evaluation of cardiac connexin43 showed a loss of immunoreactive particles among the samples of LFN-exposed rats (3). The Cx43/muscle ratio *in totum* decreased 43.3% among the LFN exposed rats ($p<0.01$). This alteration was evident at the free ventricular wall but not in the interventricular septum suggesting that this anatomical region could be more protected against the effects of LFN. Nevertheless there was a significant alteration of the electrophysiological *milieu* which suggests that LFN can lead to arrhythmogenic consequences. In fact, changes on gap junctional Cx43 have been implicated in ventricular remodeling and development of arrhythmias in several cardiac diseases (16 - 22).

Some experimental studies showed that reductions in connexin43 are associated with slowing of impulse propagation and an increase of propensity for ventricular arrhythmias (23 - 25). In addition, the reduced connexin expression in association with an increase of collagen deposition may increase the arrhythmogenicity of the heart (26).

The results of our studies showing perivascular and myocardial fibrosis (1, 2) together with a possible gap junction remodeling induced by LFN (3) made the development of a morphological arrhythmogenic substrate by LFN possible and we put forward the hypothesis of a link between LFN and ventricular tachyarrhythmias. Thus, clinical

evaluations of arrhythmogenic ventricular substrates in individuals exposed to LFN are needed to confirm these data.

Finally, new data were obtained with the evaluation of collagen and cardiomyocyte ultrastructure (4). The immunohistochemical evaluation showed significant increase for the collagen I/muscle and collagen III/muscle ratios in LFN-exposed animals and observations through TEM demonstrated high concentration of collagen in the extracellular matrix next to fibroblasts. These structural and ultrastructural changes confirm the pronounced effects of LFN on the connective tissue and suggest that the morphological substrate can have important ventricular functional consequences.

Although TEM was performed as a preliminary illustrating purpose, new alterations in cardiomyocyte ultrastructure were observed. The presence of numerous enlarged mitochodria was the organelle alteration in response to LFN suggesting that cardiac morphological changes may not be confined to the extracellular matrix. Corroborating this hypothesis are the data observed in other tissues as parotid gland, lung and gastric mucosa (27 - 30).

Another relevant alteration in cardiomyocyte ultrastructure was the observation of separated cell membranes, putting forward the hypothesis of gap junction disruption possibly caused by interstitial fibrotic development. This ultrastructural change must be confirmed in further experimental studies.

Taking into account the possible severe clinical consequences of the effects of LFN on the heart, we stressed the importance of the clinical diagnosis and characterization of myocardial fibrosis and its possible functional and arrhythmic consequences.

In this regard and although the relation between biochemical markers and myocardial fibrosis induced by LFN is not known, a potential clinical application of biomarkers is admissible, taking into consideration the significant elevation of collagen I and III observed among animals exposed to LFN and the fact that high levels of procollagens are detected in several cardiac diseases (13, 31 - 36).

In addition, echocardiography can be used to evaluate ventricular diastolic function and magnetic resonance can be utilized to quantify cardiac fibrosis, among people exposed to LFN.

In conclusion, our results showed perivascular and myocardial fibrotic development, as well as a reduction in gap junctions and defined a new cardiac morphological model induced by LFN, based on three anatomical components.

The significant changes in the extracellular matrix, together with the reduction of connexin43 and the observed ultrastructural alterations at the intercalated discs of the cardiomyocytes make possible the statement of a morphological substrate with possible ischemic, functional and arrhythmic consequences, induced by low-frequency noise.

In addition, our clinical observation for many years, of acute coronary syndromes with normal coronary arteries, worsening of ventricular diastolic function in several cardiac diseases as well as “idiopathic” ventricular tachyarrhythmias including ventricular fibrillation, can fit within the pathophysiological consequences of LFN and should be looked upon with other clinical eyes.

Thus, in modern societies, we put forward the hypothesis that LFN could be another cause for developing myocardial ischemia, heart failure and ventricular tachyarrhythmias, which opens new and promising paths for experimental and clinical research in these areas.

References

1- Antunes E, Oliveira P, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Histomorphometric evaluation of the coronary arterial vessels in rats submitted to industrial noise. Acta Cardiol 2013; 68: 285 – 289.

2- Antunes E, Oliveira P, Borrecho G, Oliveira MJR, Brito J, Águas A, Martins dos Santos J. Myocardial fibrosis in rats exposed to low frequency noise. Acta Cardiol 2013; 68: 241 – 245.

3- Antunes E, Borrecho G, Oliveira P, Brito J, Águas A, Martins dos Santos J. Immunohistochemical evaluation of cardiac connexin43 in rats exposed to low-frequency noise. Int J Clin Exp Pathol 2013; 6: 1874 – 1879.

4- Antunes E, Borrecho G, Oliveira P, Brito J, Águas A, Martins dos Santos J. Effects of low-frequency noise on cardiac collagen and cardiomyocyte ultrastructure: An immunohistochemical and electron microscopy study. Int J Clin Exp Pathol; (Accepted 2013).

5- Isoyama S, Ito N, Satoh U, Takishima T. Collagen deposition and the reversal of coronary reserve in cardiac hypertrophy. Hypertension 1992; 20:491 – 500.

6- Dai Z, Aoki T, Fukumoto Y, Shimokawa H. Coronary perivascular fibrosis is associated with impairment of coronary blood flow in patients with non-ischemic heart failure. J Cardiol 2012; 60: 416 – 421.

7- Diez J. Mechanisms of cardiac fibrosis in hypertension. J Clin Hypertens 2007; 9: 546 – 550.

8- van den Borne SWM, Diez J, Blankesteijn M, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 2010; 7: 30 – 37.

9- Pujadas S, Carreras F, Arrastio X, Leta R, Vila M, Subirana MT, Bayés-Genis A, Pons-Lladó G. Detection and quantification of myocardial fibrosis in hipertrophic cardiomyopathy by contrast enhanced cardiovascular magnetic resonance. Rev Esp Cardiol 2007; 60: 10 – 14.

10- Bruckner BA, Razeghi P, Stetson S, Thompson L, Lafuente J, Entman M, Loebe M, Noon G, Taegtmeyer H, Frazier OH, Youker K. Degree of cardiac fibrosis and hypertrophy at time of implantation predicts myocardial improvement during left ventricular assist device support. J Heart Lung Transplant 2004; 23: 36 – 42.

11- Weber KT, Brilla CG, Janicki JS. Myocardial fibrosis. Functional significance and regulatory factors. Cardiovasc Res 1993; 27: 13 – 31.

12- Hill MA, Davis MJ, Meininger GA, Potocnik SJ, Murphy TV. Arteriolar myogenic signaling mechanisms: implications for local vascular function. Clin Hemorheol Microcirc 2006; 34: 67 – 79.

13- Barasch E, Gottdiener JS, Aurigemma G, Kitzman DW, Han J, Kop WJ, Tracy RP. Association between elevated fibrosis markers and heart failure in the elderly.The cardiovascular health study. Circ Heart Fail 2009; 2: 303 – 310.

14- Assyang P, Carré F, Chevalier B, Delcayre C, Mansier P, Swing-hedouw B. Compensated cardiac hypertrophy: arrhythmogenicity and the new myocardial phenotype. I. Fibrosis. Cardiovasc Res 1997; 34: 439 – 444.

15- Chao P-C, Yeh C-Y, Juang Y-J, Hu C-Y, Chen C-J. Effect of low frequency noise on the echocardiographic parameter E/A ratio. Noise & Health 2012; 14 (59): 155 – 158.

16- Smith JH, Green CR, Peters NS, Rothery S, Severs NJ. Altered patterns of gap junction distribution in ischemic heart disease. An immunohistochemical study of human

myocardium using laser scanning confocal microscopy. Am J Pathol 1991; 139: 871 – 878.

17- Kostin S, Rieger M, Dammer S, Hein S, Richter M, Klovekorn WP, Bauer EP, Schaper J. Gap junction remodeling and altered connexin43 expression in the failing human heart. Mol Cell Biochem 2003; 242: 135 – 144.

18- Kitamura H, Ohnishi Y, Yoshida A, Okajima K, Azumi H, Ishida A, Galeano EJ, Kubo S, Hayashi Y, Itoh H, Yokoyama M. Heterogeneous loss of connexin43 protein in nonischemic dilated cardiomyopathy with ventricular tachycardia. J Cardiovasc Electrophysiol 2002; 13: 865 – 887.

19- Kitamura H, Ohnishi Y, Yoshida A, Okajima K, Azumi H, Ishida A, Galeano EJ, Kubo S, Fukuzawa K, Takano T, Yokoyama M. Correlation of connexin43 expression and late ventricular potentials in nonischemic dilated cardiomyopathy. Circ J 2003; 67: 1017 – 1021.

20- Kostin S, Dammer S, Hein S, Klovekorn WP, Bauer EP, Schaper J. Connexin 43 expression and distribution in compensated and decompensated cardiac hypertrophy in patients with aortic stenosis. Cardiovasc Res 2004; 62: 426 – 436.

21- Peters NS, Green CR, Poole-Wilson PA, Severs NJ. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. Circulation 1993; 88: 864 – 875.

22- Takeuchi S, Akita T, Takagishi Y, Watanabe E, Sasano C, Honjo H, Kodama I. Disorganization of gap junction distribution in dilated atria of patients with chronic atrial fibrillation. Circ J 2006; 70: 575 – 582.

23- Gutstein DE, Morley GE, Tammaddon H, Vaidya D, Schneider MD, Chen J, Chien KR, Stuhlmann H, Fishman GI. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circ Res 2001; 88: 333 – 339.

24- Danik SB, Liu F, Zhang J, Suk HJ, Morley GE, Fishman GI, Gutstein DE. Modulation of cardiac gap junction expression and arrhythmic susceptibility. Circ Res 2004; 95: 1035 – 1041.

25- van Rijen HVM, Eckardt D, Degen J, Theis M, Ott T, Willecke K, Jonsma HJ, Opthof T, Bakker JMT. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. Circulation 2004; 109: 1048 – 1055.

26- Jansen JA, van Veen AA, Bosch AA, van der Nagel R, Vos MA, Bakker JM, van Rijen HV. Arrhythmia vulnerability of aged haploinsufficient Cx43 mice is determinant by heterogeneous downregulation of Cx43 combined with increased fibrosis. Circulation 2008; 118: S494.

27- Grande NR, Águas AP, Sousa Pereira A, Monteiro E, Castelo Branco NAA. Morphological changes in the rat lung parenchyma exposed to low frequency noise. Aviat Space Environ Med 1999; 70: A70 – A77.

28- Oliveira PM, Pereira da Mata AD, Martins dos Santos JA, da Silva Marques DN, Branco NC, Silveira JM, Correia da Fonseca JC. Low-frequency noise effects on the parotid gland of the Wistar rat. Oral Dis 2007; 13: 468 – 73.

29-, Martins dos Santos J, Oliveira P, Laranjeira N, Aguas A, Castelo-Branco N. Noise-induced gastric lesions: a light and electron microscopy study of the rat gastric wall exposed to low frequency noise. Arq Gastroenterol 2012; 49: 82 – 88.

30- de Sousa Pereira A, Águas AP, Grande NR, Mirones J, Monteiro E, Castelo Branco NAA. The effect of chronic exposure to low frequency noise on rat tracheal epithelia. Aviat Space Environ Med. 1999; 70: A186 – A190.

31- Martos R, Baugh J, Ledwidge M, O'Loughlin C, Conlon C, Patle A, Donnelly SC, McDonald K,. Diastolic heart Failure: evidence of increased myocardial turnover linked to diastolic dysfunction. Circulation 2007; 115: 888 – 895.

32- Klappacher G, Franzen P, Haab D, Mehrabi M, Binder M, Plesch K, Pacher R, Grimm M, Pribill I, Eichler HG. Measuring extracellular matrix turnover in the serum of patients with idiopathic or ischemic dilated cardiomyopathy and impact on diagnosis and prognosis. Am J Cardiol 1995; 75: 913 – 918.

33- Lombardi R, Betocchi S, Losi MA, Tocchetti CG, Aversa M, Miranda M, D'Alessandro G, Cacace A, Ciampi Q, Chiariello M. Myocardial collagen turnover in hypertrophic cardiomyopathy. Circulation 2003; 108: 1455 – 60.

34- Sugimoto M, Masutani S, Seki M, Kajino H, Fujieda K, Senzaki H.High serum levels of procollagen type III N-terminal amino peptide in patients with congenital heart disease. Heart 2009; 95: 2023 – 2028.

35- Cicoira M, Rossi A, Bonapace S, Zanolla L, Golia G, Franceschini L, Caruso B, Marino PN, Zardini P. Independent and additional prognostic value of aminoterminal propeptide of type III procollagen circulating levels in patients with chronic heart failure. J Card Fail 2004;10: 403 – 411.

36- Querejeta R, López B, González A, Sánchez E, Larman M, Martínez Ubago JL, Díez J. Increased collagen type I synthesis in patients with heart failure of hypertensive origin: Relation to myocardial fibrosis. Circulation 2004; 110: 1263 – 1268.